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| <b>(21) International Application Number:</b> PCT/US91/03894<br><b>(22) International Filing Date:</b> 3 June 1991 (03.06.91)<br><b>(30) Priority data:</b><br>532,004                      1 June 1990 (01.06.90)                      US<br>701,544                      16 May 1991 (16.05.91)                      US<br><b>(71) Applicants:</b> REGENERON PHARMACEUTICALS, INC. [US/US]; 777 Old Saw Mill River Road, Tarrytown, NY 10591 (US). BOARD OF REGENTS THE UNIVERSITY OF TEXAS SYSTEM [US/US]; 201 West 7th Street, Austin, TX 78701 (US).<br><b>(72) Inventors:</b> BOULTON, Teri, G. ; 1217 Elwood, Irving, TX 75061 (US). COBB, Melanie, H. ; 58-59 Burgundy Road, Dallas, TX 75230 (US). YANCOPOULOS, George, D. ; 428 Sleepy Hollow Road, Briarcliff Manor, NY 10510 (US). NYE, Steven ; 423 East 90th Street, New York, NY 10128 (US). PANAYOTATOS, Nikos ; 95 Monmouth Court, Orangeburg, NY 10962 (US).  |           | <b>(74) Agent:</b> MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).<br><b>(81) Designated States:</b> AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).<br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> A FAMILY OF MAP2 PROTEIN KINASES<br><br><b>(57) Abstract</b><br><p>The present invention relates to a newly identified family of protein serine/threonine kinases which phosphorylate microtubule-associated protein 2 (MAP2). It is based, in part, on the cloning and characterization of novel MAP2 kinases designated extracellular signal-regulated kinase 1, 2, and 3 (ERK1, ERK2, ERK3) which are expressed in the central nervous system, and on the identification of another ERK family member, ERK4, with antisera. The present invention provides for recombinant nucleic acid molecules and proteins representing members of the MAP2 kinase family, and also for microorganisms, transgenic animals, and cell lines comprising recombinant MAP2 kinase molecules. In additional embodiments of the invention, the present invention provides for methods for assaying cellular factor activity, including, but not limited to, nerve growth factor activity, in which the activation of MAP2 kinase serves as an indicator of cellular factor activity. These methods may be extremely useful in screening compounds for the presence of a desired cellular factor activity. In specific embodiments, compounds which may be useful in the treatment of Alzheimer's disease, peripheral neuropathies, and diabetes may be identified using the methods of the invention.</p> |           |  |

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A FAMILY OF MAP2 PROTEIN KINASES1. INTRODUCTION

The present invention relates to a newly identified family of MAP2 protein kinases. It is based, in part, on the cloning and characterization of three MAP2 protein kinases, designated ERK1, ERK2, and ERK3, which are expressed in the central nervous system and elsewhere. The present invention provides for recombinant MAP2 kinase nucleic acids and proteins, cell lines and microorganisms comprising recombinant MAP2 kinase molecules, and bioassay methods for detecting the presence of biologically active compounds which utilize recombinant MAP2 kinase molecules.

2. BACKGROUND OF THE INVENTION

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2.1. PROTEIN KINASE CASCADES AND THE  
REGULATION OF CELL FUNCTION

A cascade of phosphorylation reactions, initiated by a receptor tyrosine kinase, has been proposed as a potential transducing mechanism for growth factor receptors, including the insulin receptor (Cobb and Rosen, 1984, Biochim. Biophys. Acta. 738:1-8; Denton et al., 1984, Biochem. Soc. Trans. 12:768-771). In his review of the role of protein phosphorylation in the normal control of enzyme activity, Cohen (1985, Eur. J. Biochem. 151:439-448) states that amplification and diversity in hormone action are achieved by two principal mechanisms, the reversible phosphorylation of proteins and the formation of "second messengers"; many key regulatory proteins are interconverted between phosphorylated and unphosphorylated forms by cellular protein kinases and certain protein phosphatases.

Some hormones appear to transmit their information to the cell interior by activating transmembrane signalling systems that control production of a relatively small

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number of chemical mediators, the "second messengers."  
These second messengers, in turn, are found to regulate  
protein kinase and phosphatase activities, thereby altering  
the phosphorylation states of many intracellular proteins,  
and consequently controlling the activity of enzymes which  
5 are regulated by their degree of phosphorylation (see  
Figure 1). The receptors for other hormones are themselves  
protein kinases or interact directly with protein kinases  
to initiate protein kinase signalling cascades. These  
series of events are believed to explain the diversity  
10 associated with the actions of various hormones (Cohen,  
1985, Eur. J. Biochem. 151:439-448; Edelman et al., 1987,  
Ann. Rev. Biochem. 56:567-613).

Insulin, like most cellular regulators, exerts its  
effects on many cellular processes through alterations in  
15 the phosphorylation state of serine and threonine residues  
within regulated proteins. Insulin exerts these effects  
via its receptor, which has intrinsic tyrosine-specific  
protein kinase activity (Rosen et al., 1983, Proc. Natl.  
Acad. Sci. U.S.A. 80:3237-3240; Ebina et al., 1985, Cell  
20 40:747-758). Of note, the proteins encoded by several  
oncogenes are also protein-tyrosine kinases. For example,  
P68<sup>gag-ros</sup>, a transmembrane transforming protein, bears  
many similarities to the insulin receptor, sharing 50%  
amino acid identity (for discussion, see Boulton et al.,  
25 1990, J. Biol. Chem. 265:2713-2719).

Nerve growth factor (NGF), a neurotrophic agent  
necessary for the development and function of certain  
central and peripheral nervous system neurons, is also  
believed to influence cellular functions, at least in part,  
30 by altering phosphorylation of intracellular proteins. It  
has been observed that NGF promotes changes in the  
phosphorylation of certain cellular proteins (discussed in  
Volonte et al., 1989, J. Cell. Biol. 109:2395-2403; Aletta  
et al., 1988, J. Cell. Biol. 106:1573-1581; Halegoua and  
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Patrick, 1980, Cell 22:571-581; Hama et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:2353-2357; Romano et al., 1987, J. Neurosci, 7:1294-1299). Furthermore, NGF appears to regulate several different protein kinase activities (Blenis and Erikson, 1986, EMBO J. 5:3441-3447; Cremins et al., 1986, J. Cell Biol. 103:887-893; Landreth and Rieser, 1985, J. Cell. Biol. 100:677-683; Levi et al., 1988, Mol. Neurobiol. 2:201-226; Mutoh et al., 1988, J. Biol. Chem. 263:15853-15856; Rowland et al., 1987, J. Biol. Chem. 262:7504-7513). Mutoh et al. (1988, J. Biol. Chem. 263:15853-15856) reports that NGF appears to increase the activities of kinases capable of phosphorylating ribosomal protein S6 (S6 kinases) in the PC12 rat pheochromocytoma cell line, a model system regularly used to study NGF function. Volonte et al. (1989, J. Cell. Biol. 109:2395-2403) states that the differential inhibition of the NGF response by purine analogues in PC12 cells appeared to correlate with the inhibition of PKN, an NGF-regulated serine protein kinase. Additionally, activators of the cyclic AMP dependent protein kinase (PKA) and protein kinase C (PKC) have been reported to mimic some but not all of the cellular responses to NGF (Levi et al., 1988, Mol. Neurobiol. 2:201-226). Miyasaka et al. (1990, J. Biol. Chem. 265:4730-4735) reports that NGF stimulates a protein kinase in PC12 cells that phosphorylates microtubule-associated protein-2. Interestingly, despite the many reports linking NGF with changes in phosphorylation of cellular proteins, analysis of a cDNA sequence encoding a subunit of the NGF receptor which is sufficient for low-affinity binding of ligand has indicated no evidence for a protein-tyrosine kinase domain in the cytoplasmic region of this low affinity receptor (Johnson et al., 1986, Cell 47:545-554).

## 2.2. MAP2 PROTEIN KINASE

Ribosomal protein S6 is a component of the eukaryotic 40S ribosomal subunit that becomes phosphorylated on multiple serine residues in response to a variety of mitogenic stimuli, including insulin, growth factors and various transforming proteins (for discussion, see Sturgill et al., 1988, *Nature* 334:715-718). Recently, an activated S6 kinase has been purified and characterized immunologically and molecularly (Ericson and Maller, 1986, *J. Biol. Chem.* 261:350-355; Ericson et al., 1987, *Mol. Cell Biol.* 7:3147-3155; Jones et al., 1988, *Proc. Natl. Acad. Sci. U.S.A.* 85:377-3381; Gregory et al., 1989, *J. Biol. Chem.* 264:18397-18401). Reactivation and phosphorylation of the S6 kinase occurs in vitro via an insulin-stimulated microtubule-associated protein-2 (MAP2) protein kinase providing further evidence for a protein kinase cascade (Sturgill, 1988, *supra*; Gregory et al., 1989, *supra*). MAP2 kinase has been observed to phosphorylate microtubule-associated protein-2 (MAP2) on both serine and threonine residues (Ray and Sturgill, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:1502-1506; Boulton et al., 1991, *Biochem.* 30:278-286). These observations suggest that key steps in insulin action involve the sequential activation by phosphorylation of at least two serine/threonine protein kinases (Sturgill et al., 1988, *Nature* 334:715-718; Gregory et al., 1989, *J. Biol. Chem.* 264:18397-18401; Ahn et al., 1990, *J. Biol. Chem.* 265:11495-11501), namely, a MAP2 kinase and an S6 kinase. The MAP2 kinase appears to be activated transiently by insulin prior to S6 kinase activation.

The MAP2 kinase phosphorylates S6 kinase in vitro causing an increase in its activity (Gregory et al., 1989, *J. Biol. Chem.* 264:18397-18401; Sturgill et al., 1988, *Nature*, 334:715-718); thus, the MAP2 kinase is a likely intermediate in this protein kinase cascade. The finding

that phosphorylation on threonine as well as tyrosine residues is required for MAP2 kinase activity (Anderson et al., 1990, *Nature*, 343:651-653) suggests that it, like many other proteins, is regulated by multiple phosphorylations. The phosphorylations may be transmitted through one or  
5 several signal transduction pathways.

In addition to stimulation by insulin, MAP2 kinase activity can be rapidly increased by a variety of extracellular signals which promote cellular proliferation and/or differentiation. In this regard, MAP2 kinase may be  
10 equivalent to pp42 (Cooper and Hunter, 1981, *Mol. Cell. Biol.* 1:165-178), a protein whose phosphotyrosine content increases following exposure to growth factors and transformation by viruses (Rossamondo et al., 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6940-6943) and activation of the  
15 v-ros oncogene (Boulton et al., 1990, *J. Biol. Chem.* 265:2713-2719). For example, MAP2 kinase activity is stimulated in: terminally differentiated 3T3-L1 adipocytes in response to insulin (Ray and Sturgill, 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:1502-1506); in post-mitotic adrenal  
20 chromaffin cells in response to signals that induce catecholamine secretion (Ely et al., 1990, *J. Cell Biol.* 110:731-742); in PC12 cells in response to nerve growth factor-induced neuronal differentiation (Volonte et al., *J. Cell Biol.* 109:2395-2403; Miyasaka et al. *J. Biol. Chem.*  
25 265:4730-4735) and in T lymphocytes (Nel et al., 1990, *J. Immunol.* 114:2683-2689). MAP2 kinase(s) are likely to play important roles in signal transduction in many different pathways and in a wide variety of cell types.

Ray and Sturgill (1988, *J. Biol. Chem.* 263:12721-  
30 12727) describes some chromatographic properties of a MAP2 kinase and reports the biochemical characteristics of the partially purified enzyme. MAP2 kinase was observed to have an affinity for hydrophobic chromatography matrices. The molecular weight of the partially purified enzyme was  
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observed to be 35,000 by gel filtration chromatography and 37,000 by glycerol gradient centrifugation. MAP2 kinase activity of chromatographic fractions was found to correlate with the presence of a 40 kDa phosphoprotein detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). MAP2 kinase was observed to have a  $K_m$  of 7  $\mu M$  for ATP, and did not appear to utilize GTP. It has been observed that MAP2 kinase requires phosphorylation on tyrosine as well as serine/threonine residues for activity. Ray and Sturgill (supra) cite several problems encountered in the purification of MAP2 kinase, most notably, the presence of contaminating kinases observed to phosphorylate MAP2 in vitro. In addition, only very small amounts of only partially purified protein were available following chromatographic preparation. As discussed supra, Rossomando et al. (1989, Proc. Natl. Acad. Sci. U.S.A. 86:6940-6943) have suggested that MAP2 kinase may be a tyrosine-phosphorylated form of pp42, a low abundance 42-kDa protein which becomes transiently phosphorylated on tyrosine after cell stimulation with a variety of mitogens. Rossomondo et al. (supra) observed that phosphorylation of pp42 and activation of MAP2 kinase occur in response to the same mitogens, that the two proteins comigrate on two dimensional polyacrylamide gels and have similar peptide maps, and that the two proteins copurify during sequential purification on anion-exchange, hydrophobic interaction and gel-filtration media.

### 3. SUMMARY OF THE INVENTION

The present invention relates to a newly identified family of protein serine/threonine kinases which phosphorylate microtubule-associated protein 2 (MAP2). It is based, in part, on the cloning and characterization of novel MAP2 kinases designated extracellular signal-regulated kinase 1, 2, and 3 (ERK1, ERK2, ERK3) which are

expressed in the central nervous system, and on the identification of another ERK family member, ERK4, with antisera. Accordingly, the term "MAP2 kinase" as used herein shall mean a member of the MAP2 family of kinases, including but not limited to ERK1, ERK2, and ERK3.

5       The present invention provides for recombinant nucleic acid molecules and proteins representing members of the MAP2 kinase family, and also for microorganisms, transgenic animals, and cell lines comprising recombinant MAP2 kinase molecules. In additional embodiments of the invention, the  
10       present invention provides for methods for assaying cellular factor activity, including, but not limited to, nerve growth factor activity, in which the activation of MAP2 kinase serves as an indicator of cellular factor activity. These methods may be extremely useful in  
15       screening compounds for the presence of a desired cellular factor activity. In specific embodiments, compounds which may be useful in the treatment of Alzheimer's disease, peripheral neuropathies, and diabetes may be identified using the methods of the invention.

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#### 4. DESCRIPTION OF THE FIGURES

FIGURE 1. Schematic diagram of the relationship between hormone binding to a cellular receptor and consequent changes in the phosphorylation of proteins.

25       FIGURE 2. A. SDS-PAGE analysis of final Q-Sepharose #2 fractions isolated from NGF-treated or control PC12 cells. Aliquots of fractions obtained from the final purification column (Q-Sepharose#2) were concentrated and then analyzed via 15% SDS-PAGE. Note that  
30       fractions containing the most MAP2 kinase activity contained a prominent band (arrow) with a molecular weight of approximately 43,000 kD, as described for the insulin-stimulated MAP2 kinase BSA. Ovalbumin and  
35       cytochrome C are presented as size standards.

B. The complete nucleotide sequence (SEQ ID NO:1) of the ERK1 cDNA and its predicted protein product (SEQ ID NO:2). Asterisks denote the residues most conserved among all protein kinases. The sequences of the nine tryptic peptides that were sequenced are underlined. All residues precisely determined by the amino acid sequencing matched the cDNA encoded protein sequence; questionable residues were verified from the cDNA encoded protein sequence. The fourth and seventh peptides indicated represented the minor peptide components described in the text.

FIGURE 3. Nucleotide and predicted protein sequences of ERK2 and ERK3 cDNAs and ERK1 $\psi$  pseudogene. Initiation and termination codons are boxed; approximate locations of protein kinase subdomains indicated by roman numerals; asterisks denote residues most conserved among all protein kinases (Hanks et al., 1988, Science 241:42-52); and pound signs denote which of these residues are not conserved in the indicated sequences.

A. Nucleotide (SEQ ID NO:3) and predicted protein (SEQ ID NO:4) sequence of one of the two ERK2 cDNA clones; protein coding region of the other ERK2 cDNA matches exactly, although sequences in the flanking regions diverged.

B. Complete nucleotide (SEQ ID NO:5) and predicted protein (SEQ ID NO:6) sequence of one of two ERK3 cDNA clones analyzed; sequence of the other ERK3 cDNA matches exactly although there were differences in the amounts of flanking sequence.

C. Alignment of partial sequence of ERK1 $\psi$  with the ERK1 nucleotide sequences; only amino acid differences (including the premature termination codon of ERK1 $\psi$ , which is boxed) from the ERK1 protein sequence are

indicated. Dashes indicate deletions in both the nucleotide and amino acid sequences.

FIGURE 4. Comparison of ERKs with FUS3, KSS1 and human cdc2 protein sequences.

5 A. Computer-generated alignments (MacVector Computer Analysis Software, International Biotechnologies, Inc., New Haven, CT) were visually optimized. Roman numerals indicate subdomains conserved in protein kinases (Hanks et al., 1988, Science 241:42-52). Dots indicate identity to ERK1 sequence, dashes indicate spaces  
10 introduced to improve sequence alignments.

B. Percent identities between the sequences aligned in A, determined over the length of the cdc2+sequence; mismatches, insertions and deletions between two sequences all weighted equally.

15 FIGURE 5. Use of ERK1-, ERK2- and ERK3-specific probes provides evidence for additional ERK genes.

A. Specificity of each of the ERK probes (described in Materials and Methods) was demonstrated by hybridizing  
20 three triplicate Southern blots, each with linearized plasmids containing the ERK1, ERK2 and ERK3 cDNA inserts (as marked for each lane), with each of the ERK probes as indicated below the blots.

B. Probing of Southern blots containing EcoR1-digested rat and human genomic DNA with each of the ERK-specific  
25 probes; sizes of DNA fragments indicted in kilobases.

C. Probing of Southern blots containing rat genomic DNA digested with Bgl2, BamH1 and Hind3 with each of the ERK-specific probes; sizes of DNA fragments indicated in kilobases.

30 FIGURE 6. Independent regulation of ERK transcripts in tissues, developmentally, in cultured astroglia and in the P19 embryocarcinoma cell line.

A. Distinct patterns of expression for each of the ERKs within adult nervous system, in adult peripheral  
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tissues, and in placenta. Specific probes for each of the ERKs (see Figure 3) were hybridized to Northern blots containing 10  $\mu$ g of RNA from the indicated adult tissues and brain regions. ADR, adrenal; RET, retina; SC.N., sciatic nerve; S.C., spinal cord; A.BR, adult brain; CBL, cerebellum; HBR, hindbrain; MBR, midbrain, DIEN, diencephalon; STR, striatum; HIP, hippocampus; CTX, neocortex; OLF, olfactory bulb; SKIN, skin; HRT, heart; MUS, muscle; LUNG, lung; INT, intestine; KID, kidney; LIV, liver; SPL, spleen; THY, thymus; PLAC, placenta.

B. ERK transcripts are developmentally regulated within the nervous system and in peripheral tissues. Ten  $\mu$ g of total RNA isolated from the indicated developmental stages (E: embryonic day; P: post-natal day; AD: adult) of rat brain, spinal cord, hippocampus (HIP), liver and heart were compared for hybridization to each of the ERK-specific probes.

C. ERK2 and ERK3 transcripts expressed at low levels in cultured astroglia. Ten micrograms of total RNA from adult rat brain (BRN) or cultured astroglia (AST) probed with each of the ERK specific probes, as indicated.

D. Independent regulation of individual ERK genes during differentiation of P19 embryocarcinoma cells. Ten micrograms of total RNA from adult rat brain or from undifferentiated P19 cell (STEM), retinoic acid-induced (NEUR) or DMSO-induced (MUSC) were used to prepare replicate Northern blots which were probed as indicated. LANGFR signifies a probe for the low-affinity NGF receptor, the GAPDH control probe verifies that equal amounts of RNA were loaded in each lane.

FIGURE 7. Expression of active ERK2 in E. coli.



- A. Silver stained gels of equal amounts of protein from lysates of *E. coli* expressing ERK2 or vector alone. The arrow denotes recombinant ERK2.
- B. Immunoblot with antiserum 837 of the same amount of *E. coli* extracts shown in A and about 40 ng of partially purified ERK1.
- C. Silver stain (left) and autophosphorylation (right) of 162, 270, or 540 ng of purified recombinant ERK2.
- D. Kinase activity of purified recombinant ERK2 incubated for 0, 15, 30, 45, and 60 minutes with MBP.

10 FIGURE 8. Specificity of antipeptide antibodies.

- A. Coomassie blue stain of 100  $\mu$ g of soluble protein from PC12 cells and adult rat brain.
- B. Immunoblot of partially purified ERK1, recombinant ERK2, and 100  $\mu$ g of soluble protein from PC12 cells, 100  $\mu$ g of soluble (s) and particulate (p) protein from embryonic brain (EM BR) and adult brain (AD BR) (prepared as described in Boulton et al., 1991, Biochem. 30:278-286) with antiserum 956.
- C. Duplicate blot probed with antiserum 837.

20 FIGURE 9. Immunoprecipitation of  $^{32}$ P-labeled ERK proteins from insulin-stimulated Rat 1 HIRc B cells and NGF-stimulated PC12 cells.

- A. ERK1 was immunoprecipitated with antiserum 837 from  $^{32}$ P-labeled (left) Rat 1 HIRc B cells with (+) or without (-) exposure to insulin and (right) PC12 cells with (+) or without (-) exposure to NGF. Tick marks indicate molecular weight standards of 116, 84, 58, 48.5, 36.5, and 26.6 kDa.
- B. As in part A with and without NGF but with denaturing immunoprecipitation.
- C. Phosphoamino acid analysis of immunoprecipitated ERK1 from NGF-treated PC12 cells. The positions of the phosphoamino acid standards are noted. After 4 hours

of labeling, ERK1 was only phosphorylated on serine in the absence of NGF.

FIGURE 10. Immunoblot of immunoprecipitated ERK proteins.

ERKs were immunoprecipitated from 1 mg of supernatant protein from insulin-treated (+) or untreated (-) Rat 1 HIRc B cells under denaturing conditions using antiserum 837. The immunoprecipitated proteins were resolved by SDS-PAGE and probed with either antibodies to phosphotyrosine (P-Y) or with ERK antiserum 691. Lanes labeled ERK contain an aliquot of a phenyl-Sepharose fraction containing both ERKs 1 and 2.

FIGURE 11. Chromatography of supernatants from NGF-treated or untreated PC-12 cells on Mono Q. 10 mg of protein from supernatants of PC12 cells either untreated or treated with NGF were chromatographed on a Mono Q column. Kinase activity with MBP is shown in the upper panel. Numbered fractions were precipitated and immunoblotted with the indicated antibody, either 956, 837 or antiphosphotyrosine ( $\alpha$ P-Y).

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#### 5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention will be divided into the following subsections:

- (i) cloning of the MAP2 kinase protein;
- (ii) identification of additional members of the MAP2 protein kinase family;
- (iii) expression of recombinant MAP2 protein kinase;
- (iv) generation of anti-MAP2 protein kinase antibodies;
- (v) bioassays for MAP2 kinase activation; and
- (vi) utility of the invention.

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### 5.1. CLONING OF THE MAP2 PROTEIN KINASE

According to the present invention, MAP2 protein kinase may be cloned by identifying cloned nucleic acids which contain sequences homologous to known MAP2 kinase sequence, for example, but not limited to, the sequences  
5 set forth in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3), and 3B (SEQ ID NO:5), and/or contained in plasmids pBS-rERK1, pBS-rERK2, or pBS-rERK3, as deposited with the ATCC and assigned accession numbers 40808, 40809, and \_\_\_\_\_, respectively. Alternatively, it may be desirable to obtain  
10 such sequence information from purified MAP2 kinase protein.

Purified MAP2 kinase may be obtained from tissues which contain MAP2 kinase activity, including, but not limited to, T lymphocytes, insulin-treated, terminally  
15 differentiated 3T3-L1 adipocytes, post-mitotic adrenal chromaffin cells induced to secrete catecholamines, PC12 cells treated with nerve growth factor, brain tissue, or insulin-treated rat 1 HIRc B cells, as well as lower eukaryotes such as sea star and Xenopus laevis oocytes.  
20 Purification of MAP2 kinase from PC12 cells appears to parallel purification of MAP2 kinases from insulin treated rat 1 HIRc B cells (FIGURE 2A).

In a specific embodiment of the invention, and not by way of limitation, MAP2 kinase may be purified to a large  
25 extent, as follows (Boulton et al., 1991, Biochem. 30:278-286). Cells containing MAP2 kinase may be used to prepare a cell free extract comprising a crude preparation of MAP2 kinase. For example, either PC12 cells may be cultured in DME medium containing 10% fetal bovine serum and 5% horse  
30 serum, and then, prior to NGF treatment, may be incubated in serum-free medium for about one hour. NGF at a concentration of about 4 nM may then be added, and the cells may be incubated for 5 minutes. Alternatively, insulin-treated Rat 1 HIRc B cells may be used. The medium  
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may then be removed and the cells rinsed and scraped into iced homogenization solution that contains 20 mM p-nitrophenylphosphate, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 50 mM sodium fluoride, 50  $\mu$ M sodium orthovanadate and 5 mM benzamidine (MAP2 kinase). Equal numbers of dishes of untreated cells may desirably be harvested as controls. All further steps are preferably performed at 4°C. Cells may be broken with 30-50 strokes of a Dounce homogenizer and homogenates may be centrifuged at 4000 x g for 5 minutes. The supernatants may then be centrifuged at 97,000 x g for 60 minutes. The resulting supernatants may then be assayed, preferably immediately, then frozen in liquid nitrogen.

For purification of MAP2 kinase, soluble fractions (225-300 ml) combined from 150 to 200 150-cm<sup>2</sup> dishes of insulin-treated Rat 1 HIRc Bell cells may be adjusted to a conductivity of 3.5 mS (with water) and to concentrations of 40  $\mu$ M cAMP, 0.5 mM phenylmethylsulfonylfluoride and 0.1  $\mu$ M pepstatin prior to chromatography on a Q-Sepharose column (1.5 x 19 cm). The column may be washed with 4 to 5 volumes of buffer A (10% glycerol, 25 mM Tris-HCl, pH 7.5, 50  $\mu$ M sodium orthovanadate, 1 mM dithiothreitol, 50 mM NaF, 20 mM  $\beta$ -glycerol phosphate, 1 mM EGTA, 10 mM benzamidine, 10 mM p-nitrophenylphosphate, 0.5 mM phenylmethylsulfonyl fluoride, and 0.1  $\mu$ M pepstatin) containing 40  $\mu$ M cAMP. Protein may then be eluted by a gradient of 0-0.4 M NaCl in buffer A. Fractions containing stimulated MAP2 kinase activity may be pooled and applied to a phenyl-Sepharose column (1.5 x 18 cm). The column may then be washed with 5 column volumes of buffer A containing 0.25 M NaCl and protein may be eluted with a descending gradient of 0.25-0.025 M NaCl plus an ascending gradient of 0-65% ethylene glycol in buffer A without glycerol. Kinase activity may be pooled from the phenyl-Sepharose column and applied directly to a 5 ml column (1.5 x 3 cm) of S-Sepharose

followed by a 5 ml column of phosphocellulose (1.5 x 3 cm). In both cases, unadsorbed material containing MAP2 kinase activity and 2 column volumes of wash may be collected. The MAP2 kinase activity from the phosphocellulose column may be applied directly to a QAE-Sepharose column (1 x 24  
5 cm). The column may be washed with 5 volumes of buffer A and protein may be eluted with a gradient of 0-0.4 M NaCl in buffer A. The fractions containing MAP2 kinase activity may then be pooled, Brij-58 may be added to give a final concentration of 0.01% (included in all subsequent steps),  
10 and the sample may be concentrated by ultrafiltration to 1.5-2 ml in order to load onto an Ultrogel ACA54 column (1 x 112 cm) equilibrated in buffer A containing 0.2 M NaCl and 0.01% Brij-58. Fractions from the gel filtration column may be collected into tubes containing 2.4 mM  
15 leupeptin. The fractions containing activity may be concentrated and diluted with 25 mM Tris, pH 7.5, 1 mM DTT, 10 mM sodium phosphate, 0.1  $\mu$ M pepstatin, 0.5 mM phenylmethyl sulfonyl fluoride containing 0.01% Brij-58 until the conductivity is reduced to 3 mS and then may be  
20 applied to DEAE-cellulose (0.7 x 18 cm). The activity may be eluted with a gradient of 0-0.25 M NaCl in buffer A. Fractions containing activity may be pooled, and, as necessary, concentrated and diluted as above to apply to either a Mono Q HR 5/5 or a Q-Sepharose (0.5 x 9 cm)  
25 column. The MAP2 kinase activity may be eluted with a gradient of 0-0.25 M NaCl (from Mono Q) in buffer A. Fractions may be assayed and then immediately frozen in liquid nitrogen.

Purified MAP2 kinase may then be digested with trypsin  
30 and the resulting peptides subjected to HPLC (Abersold et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:6970-6974) as described in section 6.1, *infra*. The peptides from one of the resulting peaks may then be subjected to a second chromatographic separation. In order to determine  
35

fragments of MAP2 kinase protein sequence accurately, it may be necessary to perform repeated purification of peptides and to discriminate between major and minor component peptides, as would be recognized by one skilled in the art.

5       Peptides may be sequenced by any method known in the art. For example, fractions containing the enzyme may be pooled and final concentrations of 0.05% Lubrol and 8.5% trichloroacetic acid (w/v) may be added to precipitate the protein. After washing with acetone, the protein may be  
10 dissolved in electrophoresis buffer and 250 pmol may be loaded onto a 10% polyacrylamide gel in SDS. Protein may be electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). The 43 kDa band may be excised for in situ digestion with trypsin (Abersold et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:6970-6974),  
15 leaving the minor component, which migrates only slightly faster, on the nitrocellulose. Peptides released from the excised piece of nitrocellulose may be subjected to HPLC on a Model 130A chromatography system (Applied Biosystems, Inc., Foster City, CA) equipped with a 2.1 x 100 mm Applied Biosystems RP-300 column. Separations may be performed in 0.1% trifluoroacetic acid at a flow rate of 50  $\mu$ l/min using a gradient of 0-70% (v/v) acetonitrile of 100-min duration. Absorbency of the eluate may be monitored at 214 nm and the  
25 components that eluted may be collected manually. Peptides may be dried onto 1 cm discs of Whatman GF/C paper and sequenced using an Applied Biosystems, Inc. Model 470A amino acid sequencer equipped with a Model 120A phenylthiohydantoin analyzer, according to manufacturer's  
30 specifications.

35       The purification of suitable amounts of MAP2 kinase protein to permit microsequencing makes possible the cloning of a MAP2 kinase cDNA. A strategy for such cloning might be to generate a complementary oligonucleotide probe,

based on a segment of known amino acid sequence, and to use this probe to screen cDNA libraries generated from tissue presumed to synthesize mRNA encoding MAP2 kinase as follows. First, the amino acid sequence derived from purified MAP2 kinase protein may be used to deduce

5 oligonucleotide primers which may be generated and used in standard screening techniques or used in polymerase chain reaction (PCR) (Saiki et al., 1985, Science 230:1350-1354). Because of the degeneracy of the genetic code, in which several triplets may specify the same amino acid, several

10 oligonucleotides should be synthesized for a given amino acid sequence, in order to provide for multiple potential nucleotide sequence combinations; the resulting oligonucleotides are referred to as degenerate primers. For example, in a specific embodiment of the invention, a

15 series of degenerate oligonucleotides may be synthesized that correspond to the coding or anti-coding strands for segments of tryptic peptide sequences obtained from purified MAP2 kinase protein. The oligonucleotides may desirably contain non-degenerate tails at their 5' ends;

20 the tail of each coding strand oligonucleotide may contain, for example, an EcoR1 restriction site, while the tail of each anti-coding strand oligonucleotide may, for example, contain a Sall restriction site. Each coding strand oligonucleotide may then be combined with each anti-coding

25 oligonucleotide in individual PCR reactions using cDNA from Rat 1 cells as template; the PCR reactions and the preparation of the genomic and cDNA templates may then be performed as described in Maisonpierre, C. et al., 1990, Science 247:1446-1451 and Bothwell, A., Yancopoulos, G. and

30 Alt, F., 1990, "Methods for Cloning and Analysis of Eukaryotic Genes", Jones and Bartlett, Boston, MA. The amplified product obtained using, for example, the QYIGEG coding oligonucleotide and the DLKPSN anti-coding oligonucleotide (designated QYDL) may then be isolated

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using a Sephadex G-50 spin column, digested with EcoR1 and Sall, gel purified using 2% Nusieve (FMC Bioproducts), and subcloned into a vector comprising suitable restriction sites, such as the pGEM4Z vector (Promega).

5 A suitable library, believed to be likely to contain a MAP2 kinase gene, may then be screened with labeled nucleic acid probe (for example, subcloned PCR product radiolabeled using a PCR-based protocol (Maisonpierre et al., 1990, Science 247:1446-1451)). Examples of a suitable library  
10 would include a rat brain or T lymphocyte cDNA library or a cDNA library produced from PC12 cells or post-mitotic adrenal chromaffin cells, to name but a few. Hybridization conditions may be performed as described in Maisonpierre et al. (1990, Science 247:1446-1451) or using any standard techniques; washing of filters may preferably be performed  
15 first at low stringency (2 X SSC (20 mM sodium citrate, pH 7.0, 0.15 M NaCl), 0.1% SDS at 60°C) and then at high stringency (0.2 X SSC, 0.1% SDS at 60°C).

Once obtained, a MAP2 kinase gene may be cloned or subcloned using any method known in the art. A large  
20 number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda  
25 derivatives, or plasmids such as pBR322, pUC, or Bluescript® (Stratagene) plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

30 The MAP2 kinase gene may be inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has  
35



complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. It may prove advantageous to incorporate restriction endonuclease cleavage sites into the oligonucleotide primers used in polymerase chain reaction to facilitate insertion into vectors. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and MAP2 kinase gene may be modified by homopolymeric tailing.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated MAP2 kinase gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

According to a preferred embodiment of the invention, once a cDNA-derived clone encoding MAP2 kinase has been generated, a genomic clone encoding MAP2 kinase may be isolated using standard techniques known in the art. For example, a labeled nucleic acid probe may be derived from the MAP2 kinase clone, and used to screen genomic DNA libraries by nucleic acid hybridization, using, for example, the method set forth in Benton and Davis (1977, *Science* 196:180) for bacteriophage libraries and Grunstein and Hogness (1975, *Proc. Natl. Acad. Sci. U.S.A.* 72:3961-3965) for plasmid libraries. Retrieved clones may then be analyzed by restriction-fragment mapping and sequencing techniques according to methods well known in the art.

Furthermore, additional cDNA clones may be identified from a cDNA library using the sequences obtained according to the invention.

5                   5.2.   IDENTIFICATION OF ADDITIONAL MEMBERS  
                    OF THE MAP2 PROTEIN KINASE FAMILY

The present invention provides for recombinant nucleic acid molecules corresponding to mammalian nucleic acids which are homologous to the nucleic acid sequences substantially as depicted in FIGURES 2B (SEQ ID NO:1), 3A  
10 (SEQ ID NO:3) and 3B (SEQ ID NO:5) or portions thereof of at least 10 nucleotides.

According to the present invention, by screening a DNA library (comprising genomic DNA or, preferably, cDNA) with oligonucleotides corresponding to MAP2 kinase sequence  
15 derived either from protein sequence data or from the nucleic acid sequence set forth in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5), clones may be identified which encode distinct members of the MAP2 kinase family, as exemplified in Section 7, infra, in which  
20 additional members of the MAP2 kinase family were identified. By decreasing the stringency of hybridization, the chances of identifying somewhat divergent members of the family may be increased. It may also be desirable to use sequences substantially shared by members of the MAP2  
25 kinase family which have been sequenced preferably, for example, sequences from domains V or VI; such highly conserved regions may be particularly useful in identifying additional members of the MAP2 kinase family. Library screening may be performed using, for example, the  
30 hybridization technique of Benton and Davis (1977, Science 196:180) or Grunstein and Hogness (1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961-3965). Clones identified by hybridization may then be further analyzed, and new family members may be identified by restriction fragment mapping

and sequencing techniques according to methods well known in the art.

It may be desirable to utilize polymerase chain reaction (PCR) technology (Saiki et al., 1985), Science 230:1350-1354) to identify additional members of the MAP2  
5 protein kinase family. For example, sense and antisense primers corresponding to known MAP2 protein kinase sequence (which preferably appears to be conserved among characterized members of the MAP2 protein kinase family) may be used in PCR, with cDNA obtained from cells which  
10 produce MAP2 kinase as template. It may be desirable to design these primers such that they include restriction enzyme cleavage sites which may facilitate the insertion of the products of PCR into appropriate cloning vectors. The products of PCR may be inserted into suitable vectors as  
15 set forth in Section 5.1, supra, and the resulting clones may then be screened for new family members. Such screening may be performed using standard techniques, including hybridization analysis using probes corresponding to known MAP2 kinase sequence. For example, a series of  
20 probes representing different regions of an already characterized MAP2 kinase protein may be hybridized at low stringency to duplicate filters carrying DNA from clones generated using PCR, as outlined above. It may be observed that various clones may hybridize to some probes, but not  
25 others. New family members may also be identified by increasing the stringency of the hybridization conditions, wherein new members not identical to probes derived from known members (e.g. ERK1, ERK2 or ERK3) would hybridize less strongly at higher stringency. Alternatively, new  
30 family members may be identified by restriction mapping or sequencing analysis using standard techniques to reveal differences in restriction maps or sequences relative to known family members.

### 5.3. EXPRESSION OF RECOMBINANT MAP2 PROTEIN KINASE

The present invention provides for recombinant MAP2 protein kinase molecule comprising the amino acid sequence substantially as depicted in FIGURES 2B (SEQ ID NO:2), 3A (SEQ ID NO:4) or 3B (SEQ ID NO:6), or a portion thereof, 5 which has a molecular weight, by SDS-PAGE, of between about 41 and 48 kDa, or about 62-63 kDa, or which comprises a portion homologous to the yeast FUS3 or KSS1 protein kinase as well as a short amino terminal extension or which has a carboxy terminal extension of about 180 amino acids. The 10 present invention also provides for mammalian MAP2 protein kinases homologous to the above-mentioned molecules.

In order to express recombinant MAP2 kinase, the nucleotide sequence coding for a MAP2 kinase protein, or a portion thereof, can be inserted into an appropriate 15 expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native MAP2 kinase gene and/or its flanking 20 regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); 25 microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of 30 suitable transcription and translation elements may be used.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene 35

consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence  
5 encoding MAP2 kinase protein or peptide fragment may be regulated by a second nucleic acid sequence so that MAP2 kinase protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of MAP2 kinase may be controlled by any  
10 promoter/enhancer element known in the art. Promoters which may be used to control MAP2 kinase expression include, but are not limited to, the SV40 early promoter region (Berthouille and Chambon, 1981, Nature 290:304-310), [the CMV promoter] the promoter contained in the 3' long  
15 terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature  
20 296:39-42); prokaryotic expression vectors such as the  $\beta$ -lactamase promoter (Villa-Komaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant  
25 bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control  
30 regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald,  
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- 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).
- Expression vectors containing MAP2 kinase gene inserts can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to an inserted MAP2 kinase gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence

of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the MAP2 kinase gene is inserted within the

5 marker gene sequence of the vector, recombinants containing the MAP2 kinase insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the

10 recombinant. Such assays can be based, for example, on the physical or functional properties of the MAP2 kinase gene product in bioassay systems as described supra, in Section 5.2. However, if cells containing MAP2 kinase expression constructs contain intrinsic MAP2 kinase, activity

15 resulting from the construct can be distinguished from endogenous kinase activity (e.g. put a distinguishing tag on the recombinant molecule) or by subtracting background levels of endogenous kinase.

Once a particular recombinant DNA molecule is

20 identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors

25 which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors,

30 to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be

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elevated in the presence of certain inducers; thus, expression of the genetically engineered MAP2 kinase protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and  
5 modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated  
10 core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of the heterologous MAP2 kinase protein. Furthermore, different vector/host expression systems may effect processing reactions such as  
15 proteolytic cleavages to different extents.

Once a recombinant which expresses the MAP2 kinase gene is identified, the gene product should be analyzed. This can be achieved by assays based on the physical or functional properties of the product.

20 Once the MAP2 kinase protein is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the  
25 purification of proteins.

The presence of functional MAP2 kinase activity may be determined as set forth in section 5.5, infra.

#### 5.3.1. MAP2 GENE KINASE GENES AND PROTEINS

30 Using the methods detailed supra and in Example Sections 6 and 7, infra, the following nucleic acid sequences were determined, and their corresponding amino acid sequences deduced. The sequences of two rat MAP2  
35 kinase cDNAs were determined, and are depicted in FIGURES



2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5). Each of these sequences, or their functional equivalents, can be used in accordance with the invention. Additionally, the invention relates to MAP2 kinase genes and proteins isolated from porcine, ovine, bovine, feline, 5 avian, equine, or canine, as well as primate sources and any other species in which MAP2 kinase activity exists. The present invention also provides for ERK4, as identified and described in Section 7, infra, which corresponds to a protein having a molecular weight of about 45 kDa. The 10 invention is further directed to homologous subsequences of MAP2 kinase nucleic acids comprising at least ten nucleotides, such subsequences comprising hybridizable portions of the MAP2 kinase sequence which have use, e.g., in nucleic acid hybridization assays, Southern and Northern 15 blot analyses, etc. The invention also provides for MAP2 kinase proteins, fragments and derivatives thereof, according to the amino acid sequences set forth in FIGURES 2B (SEQ ID NO:2), 3A (SEQ ID NO:4) and 3B (SEQ ID NO:6) or their functional equivalents and for proteins homologous to 20 such proteins, such homology being of at least about 30 percent. The invention also provides fragments or derivatives of MAP2 kinase proteins which comprise antigenic determinant(s) or which are functionally active or which are at least six amino acids in length. As used 25 herein, functionally active shall mean having the capacity to phosphorylate MAP2 or other relevant substrates (e.g. MBP, S6 kinase; see Section 5.5, infra).

For example, the nucleic acid sequences depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID 30 NO:5) can be altered by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in FIGURES 2B (SEQ 35

ID NO:2), 3A (SEQ ID NO:4) and 3B (SEQ ID NO:6) may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the MAP2 kinase genes depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5) which  
5 are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the MAP2 kinase proteins, or fragments or derivatives thereof, of the invention include, but are not limited to,  
10 those containing, as a primary amino acid sequence, all or part of the amino acid sequence substantially as depicted in FIGURES 2B (SEQ ID NO:2), 3A (SEQ ID NO:4) and 3B (SEQ ID NO:6) including altered sequences in which functionally equivalent amino acid residues are substituted for residues  
15 within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino  
20 acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids  
25 include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of  
30 the invention are MAP2 kinase proteins or fragments or derivatives thereof which are differentially modified during or after translation, e.g., by phosphorylation, glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. For example, it  
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may be desirable to modify the sequence of a MAP2 kinase such that specific phosphorylation, i.e. serine threonine, is no longer required or as important.

In addition, the recombinant MAP2 kinase encoding nucleic acid sequences of the invention may be engineered  
5 so as to modify processing or expression of MAP2 kinase. For example, and not by way of limitation, a signal sequence may be inserted upstream of MAP2 kinase encoding sequences to permit secretion of MAP2 kinase and thereby facilitate harvesting or bioavailability.

10 Additionally, a given MAP2 kinase can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to  
15 facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

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#### 5.4. GENERATION OF ANTI-MAP2 PROTEIN KINASE ANTIBODIES

According to the invention, MAP2 kinase protein, or fragments or derivatives thereof, may be used as immunogen  
25 to generate anti-MAP2 kinase antibodies. By providing for the production of relatively abundant amounts of MAP2 kinase protein using recombinant techniques for protein synthesis (based upon the MAP2 kinase nucleic acid sequences of the invention), the problem of limited  
30 quantities of MAP2 kinase has been obviated.

To further improve the likelihood of producing an anti-MAP2 kinase immune response, the amino acid sequence of MAP2 kinase may be analyzed in order to identify portions of the molecule which may be associated with

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increased immunogenicity. For example, the amino acid sequence may be subjected to computer analysis to identify surface epitopes which present computer-generated plots of hydrophilicity, surface probability, flexibility, antigenic index, amphiphilic helix, amphiphelic sheet, and secondary  
5 structure of MAP2 kinase. Alternatively, the deduced amino acid sequences of MAP2 kinase from different species could be compared, and relatively non-homologous regions identified; these non-homologous regions would be more likely to be immunogenic across various species.

10 For preparation of monoclonal antibodies directed toward MAP2 kinase, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein  
15 (1975, *Nature* 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in "Monoclonal Antibodies and Cancer Therapy,"  
20 Alan R. Liss, Inc. pp. 77-96) and the like are within the scope of the present invention.

The monoclonal antibodies for therapeutic use may be human monoclonal antibodies or chimeric human-mouse (or  
25 other species) monoclonal antibodies. Human monoclonal antibodies may be made by any of numerous techniques known in the art (*e.g.*, Teng et al., 1983, *Proc. Natl. Acad. Sci. U.S.A.* 80:7308-7312; Kozbor et al., 1983, *Immunology Today* 4:72-79; Olsson et al., 1982, *Meth. Enzymol.* 92:3-16). Chimeric antibody molecules may be prepared containing a  
30 mouse antigen-binding domain with human constant regions (Morrison et al., 1984, *Proc. Natl. Acad. Sci. U.S.A.* 81:6851, Takeda et al., 1985, *Nature* 314:452).

Various procedures known in the art may be used for  
35 the production of polyclonal antibodies to epitopes of MAP2

kinase. For the production of antibody, various host animals can be immunized by injection with MAP2 kinase protein, or fragment or derivative thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and, Corynebacterium parvum.

A molecular clone of an antibody to a MAP2 kinase epitope can be prepared by known techniques. Recombinant DNA methodology (see e.g., Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to construct nucleic acid sequences which encode a monoclonal antibody molecule, or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high performance liquid chromatography), or a combination thereof, etc.

The present invention provides for antibody molecules as well as fragments of such antibody molecules.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')_2$  fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$  fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

### 5.5. BIOASSAYS FOR MAP2 KINASE ACTIVITY

The activity of MAP2 kinase may be measured using any suitable kinase assay known in the art. For example, and not by way of limitation, the method described in (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719) as follows.

- 5 The assay for phosphorylation of MAP2 may contain 30 mM Hepes, pH 8, 50  $\mu$ M ATP (1-50 cpm/fmol), 1 mM dithiothreitol, 1 mM benzamidine, 10 mM  $MgCl_2$ , 100  $\mu$ g/ml bovine serum albumin, 3  $\mu$ g MAP2 and no more than about 10  $\mu$ g sample protein in a final volume of 30  $\mu$ l for 10 minutes  
10 at 30°C. The amount of MAP2 in the assay (100  $\mu$ g/ml) may be chosen for convenience of analysis both by SDS-PAGE and by precipitation. The enzyme is not saturated with substrate even at 1.36 mg MAP2/ml. However, with 100  $\mu$ g MAP2/ml enzyme activity may be expected to be linear with  
15 time for at least 30 minutes. All samples except for unfractionated supernatants may be routinely assayed as above in the presence of 1 mg of bovine serum albumin. Assays may be terminated by the addition of 10% trichloroacetic acid and precipitates may be collected on  
20 glass fiber filters. All assays except as noted above may be terminated by the addition of 0.25 volume of 0.3 M Tris-HCl, pH 6.9, containing 2 M mercaptoethanol, 50% glycerol and 10% SDS and analyzed by electrophoresis in SDS using 5% (MAP2) polyacrylamide gels. The gels may be  
25 stained with Coomassie blue, destained in 10% methanol and 10% acetic acid, dried and subjected to autoradiography at -80°C using Kodak XS-5 or BB-5 film with Dupont Quanta III intensifying screens. Substrate bands may be excised from gels and  $^{32}P$  may be quantitated using liquid scintillation  
30 counting.

### 5.6. UTILITY OF THE INVENTION

The present invention may be utilized to provide unique model systems for the study of mechanisms of

hormones and other cellular factors, and may also be used in methods for screening compounds for hormone/cellular factor activity and to identify agents which function as agonists or antagonists.

According to various embodiments of the invention,  
5 recombinant MAP2 kinase molecules can be used to create novel model systems for the study of mechanisms of hormones and other cellular factors. For example, and not by way of limitation, the recombinant molecules of the invention can be incorporated into cells or organisms such that higher  
10 than normal amounts of MAP2 kinase are produced, so that the effects of hyperactivation of MAP2 kinase may be evaluated. Overproduction of MAP2 kinase may identify aspects of the hormonal/cellular factor response related to MAP2 kinase activity, particularly when evaluated in  
15 comparison to cells or organisms which produce normal amounts of MAP2 kinase.

Alternatively, recombinant MAP2 kinase molecules may be engineered such that cells or organisms comprising the recombination molecules produce a mutant form of MAP2  
20 kinase which may, for example, lack the serine/threonine kinase activity of normal MAP2 kinase. The mutant kinase may, on a concentration basis, overshadow, or titrate out, the effects of normal MAP2 kinase and thereby create cells or organisms with a functional aberrancy of MAP2 kinase  
25 function. It is also envisioned that such mutant nucleic acid sequences may result in mutation of the endogenous MAP2 kinase gene, for example, by homologous recombination, creating true MAP2 kinase mutants. In light of the high levels of expression of MAP2 kinase encoding mRNA in the  
30 central nervous system, and the role of MAP2 in forming neurofibrillary tangles, it may be possible to generate a transgenic non-human animal which expresses a mutant MAP2 kinase molecule in its central nervous system (e.g. via a brain-specific promoter sequence) and which may serve as an  
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animal model system for neurological disorders such as Alzheimer's disease or for peripheral neuropathies.

In addition, because the present invention enables the production of large amounts of purified MAP2 kinase for the first time, it allows for the production of anti-MAP2  
5 kinase antibodies. Anti-MAP2 kinase antibodies, polyclonal or monoclonal, may be used in experiments utilizing cells or organisms which study the effects of selective neutralization of MAP2 kinase function. Such experiments may further elucidate the specific role of MAP2 kinase in  
10 hormone or cellular factor action.

An important embodiment of the present invention relates to methods for the screening of compounds for hormone or cellular factor activity. In specific  
15 embodiments, the present invention provides for a method of detecting the presence of a compound having nerve growth factor-like activity comprising (i) culturing cells that produce an MAP2 protein kinase (which is activated by nerve growth factor) in the presence of a compound suspected of  
20 having nerve growth factor-like activity (construed to mean activity similar but not necessarily identical to NGF, including, for example, the ability to support the growth of sympathetic neurons in culture) and (ii) detecting changes in the levels of MAP2 protein kinase activity, wherein an increase in activity is indicative of the  
25 presence of nerve growth factor-like activity. Similarly, in another specific embodiment, the present invention provides for a method of detecting the presence of a compound having insulin-like activity comprising (i) culturing cells that produce an MAP2 protein kinase (which  
30 is activated by insulin) in the presence of a compound suspected of having insulin-like activity (construed to mean activity similar but not necessarily identical to insulin, including for example, the ability to activate MAP2 kinase in insulin, responsive cells) and (ii)  
35



detecting changes in the levels of MAP2 protein kinase activity, wherein an increase in activity is indicative of the presence of insulin-like activity. The present invention therefore provides a powerful method for identifying compounds that may be useful in the treatment  
5 of diabetes. The present invention also provides for analogous methods which screen for the activity of other hormones or cellular factors. In additional embodiments of the invention, it may be desirable, in the above-mentioned screening methods, to utilize cell lines which comprises a  
10 recombinant nucleic acid molecule encoding a mammalian MAP2 kinase, including, but not limited to, recombinant nucleic acid molecules comprising sequences substantially as depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and  
15 3B (SEQ ID NO:5). Such cell lines may preferably express elevated levels of MAP2 kinase, and would therefore provide a more sensitive assay for MAP2 kinase activation. The present invention also provides for similar methods, in which cells utilized for screening comprise a recombinant nucleic acid sequence homologous to the sequence  
20 substantially as depicted in FIGURES 2B (SEQ ID NO:1), 3A (SEQ ID NO:3) and 3B (SEQ ID NO:5) or a portion thereof. The methods of the invention may be used to identify compounds that may be effective in the treatment of peripheral neuropathies or which may promote nerve  
25 regeneration. Furthermore, because NGF-responsive cholinergic neurons of the basal forebrain nucleic are consistently affected in early stages of Alzheimer's disease, the methods of the present invention may be particularly useful in identifying compounds with NGF-like  
30 activity which may be effective in the treatment of Alzheimer's disease. In addition, such methods may enable the identification of molecules capable of bypassing the hormone/receptor interaction. It may be clinically useful to inhibit the activity of MAP2 kinase in an organism,  
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using, for example, small molecules such as purine analogues.

In further embodiments of the present invention, recombinant MAP2 kinase may be used to identify other molecules, such as kinases related to cellular factor or hormone action. For example, recombinant MAP2 kinase could be used to identify additional kinases by affinity purification, wherein a MAP2 kinase may be used to adhere to other kinases which participate in a MAP2 associated phosphorylation cascade. Sequenced portions of the NGF receptor are likely to be physically associated with an as yet unidentified protein kinase. Recombinant MAP2 kinase may be useful in studying such interactions.

In another embodiment, detecting a change in a MAP2 protein kinase activity resulting from culturing cells in the presence of a compound known to or suspected to affect MAP2 protein kinase activity, can be used to detect the presence or measure the amount of such a compound and its ability to modulate MAP2 kinase activity levels. Such an effect on MAP2 kinase activity can occur directly or indirectly (e.g. through a signal transduction pathway). In a specific example of such an embodiment, the presence of a neurotrophin molecule (including but not limited to NGF, brain derived neurotrophic factor, neurotrophin-3 (NT-3) and other members of the NGF/BDNF/NT-3 family of molecules) can be detected by detecting an increase in the activity of a MAP2 protein kinase upon culturing the cells in the presence of a sample suspected of containing such a neurotrophin molecule. The cells which are cultured in such assays should express receptors for the neurotrophin molecule being detected, which receptors can be endogenous or recombinant.

6. EXAMPLE: MOLECULAR CLONING OF AN INSULIN-STIMULATED MAP2 PROTEIN KINASE: HOMOLOGY IN PHEROMONE-REGULATED CELL CYCLE CONTROL

6.1. MATERIALS AND METHODS

6.1.1. CELL LINES

5 Rat 1 HIRc B cells (McClain et al., 1987, J. Biol. Chem. 262:14663-14671) were obtained from Don McClain (Veterans Administration Medical Center, San Diego, CA). Porcine insulin was a gift from Mary Root (Eli Lilly). Restriction enzymes were obtained from New England Biolabs.

10

6.1.2. PURIFICATION AND SEQUENCING OF TRYPTIC PEPTIDES FROM MAP2 KINASE

15 MAP2 kinase was purified from insulin-treated Rat 1 HIRc B cells (Boulton et al., 1990, Biochem. 30:278-286), digested with trypsin and the resulting peptides subjected to HPLC (Abersold et al., 1987, Proc. Natl. Acad. U.S.A. 84:6970-6974). The peptides from one of the resulting peaks were subjected to a second chromatographic separation. Amino acid sequence was obtained from seven distinct peaks. One peak contained a mixture of three  
20 peptides, with one major and two minor components; the sequence of the major peptide was determined based on recovery, but the assignment of the amino acids in the minor components to their respective peptide sequences was based on the cDNA sequence (see below).

25

6.1.3. CLONING AN AMPLIFIED FRAGMENT OF THE MAP2 KINASE cDNA

30 A series of degenerate oligonucleotides were synthesized that corresponded to the coding or anti-coding strands for fragments of the tryptic peptide sequences obtained. The oligonucleotides contained non-degenerate tails at their 5' ends; the tail of each coding strand oligonucleotide had an EcoR1 restriction site, while the tail of each anti-coding strand oligonucleotide contained a

35

Sall restriction site. Each coding strand oligonucleotide was combined with each anti-coding oligonucleotide in individual PCR reactions using rat genomic DNA or cDNA from Rat 1 cells as template; the PCR reactions and the preparation of the genomic and cDNA templates were  
5 performed as described in Maisonpierre et al. (1990, Science 247:1446-1451) and Yancopoulos and Alt (1990, in "Methods for Cloning and Analysis of Eukaryotic Genes", Jones and Bartlett, Boston, MA). The amplified product obtained using the QYIGEG coding oligonucleotide and the  
10 DLKPSN anti-coding oligonucleotide (designated QYDL) was isolated using a Sephadex G-50 spin column, digested with EcoRI and Sall, gel purified using 2% Nusieve (FMC Bioproducts), and subcloned into the pGEM4Z vector (Promega).

15

#### 6.1.4. SCREENING THE cDNA LIBRARY

600,000 plaques from a rat brain cDNA library constructed in the Lambda Zap 2 vector (Stratagene) were screened using the subcloned QYDL PCR product as probe; the  
20 probe was radiolabeled using a PCR-based protocol described in Maisonpierre et al. Hybridization conditions have been described in Maisonpierre et al.; after hybridization the library filters were first washed at low stringency (2 X SSC, 0.1% SDS at 60°C) and then at high stringency (0.2 X  
25 SSC, 0.1% SDS at 68°C).

#### 6.1.5. DNA SEQUENCING

Sequencing was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977, Proc. Natl.  
30 Acad. Sci. U.S.A. 74:5463-5467), with the Sequenase Kit (version 2.0) and recommended protocols (U.S. Biochemical). All sequence was verified by sequencing both strands of the DNA, using appropriate oligonucleotides corresponding to MAP2 kinase sequence or flanking plasmid sequence.

35

#### 6.1.6. NORTHERN ANALYSIS

RNA isolation, Northern blotting, and hybridization to labeled probes were performed as described in Maisonpierre et al. (1990, Science 247:1446-1451).

5

#### 6.2. RESULTS

Purified MAP2 kinase isolated from insulin-treated rat 1 HIRc B cells consists of one major band of  $M_r=43,000$ . SDS polyacrylamide gel electrophoresis of final Q Sepharose #2 fractions isolated from NGF-treated or control PC12 10 cells indicated that purification of MAP2 kinase from PC12 cells appears to parallel purification of MAP2 kinases from insulin treated rat HIRc B cells (Figure 2A). Following tryptic cleavage, amino acid sequences of seven tryptic peptides were obtained from the 43 kd band; these peptides 15 are underlined in FIGURE 2. None of the peptides are contained in proteins in the Genbank data base. However, consensus sequences characteristic of serine/threonine protein kinases (Hanks et al., 1988, Science 241:42-52), GEGAYG (part of the nucleotide binding site) and DLKPSN 20 were found among the tryptic peptides isolated from this protein.

Degenerate oligonucleotides corresponding to several different regions of the resulting amino acid sequence were utilized in PCR reactions. Oligonucleotides corresponding 25 to segments of the peptides containing the conserved GEGAYG and DLKPSN sequences to segments most clearly yielded amplified fragments of an expected size using cDNA templates prepared from Rat 1 fibroblasts. Based on homologies to other protein kinases it was assumed that the 30 GEGAYG sequence is closer to the N-terminus of the protein and DLKPSN is closer to the C-terminus of the protein; these sequences are separated by about 120 amino acids in most protein kinases. PCR reactions using the degenerate oligonucleotide encoding the amino acids QYIGEG (SEQ ID

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No:7) (5'-

TTCTAGAATTCCA(A,G)TA(C,T)AT(A,T,C)GG(A,T,C,G)GA(A,G)GG-3',  
SEQ ID NO:8) and the degenerate oligonucleotide  
corresponding to the anti-coding strand for the amino acids  
DLKPSN (SEQ ID NO:9) (5'-

5 TTCTCGAGTCGAC(A,G)TT(A,T,C,G)GA(A,T,C,G)GG(C,T)TT(A,T,C,G)A(  
A,G)A,G)TC-3', SEQ ID NO:10) yielded an amplified product  
of approximately 360 bp. The PCR product was subcloned and  
sequenced to confirm its identity as a novel protein  
kinase. The PCR product was then used as a probe to screen  
10 a rat brain cDNA library. A single clone that hybridizes  
at high stringency has been isolated; this clone contains a  
1.9 kb cDNA insert. Northern blot analysis revealed a band  
corresponding to a mRNA of 1.9 kb, indicating that the  
insert corresponded to a full-length clone.

15 The cDNA insert has a single long open reading frame  
that could encode a protein of at least 367 residues with  
 $M_r$  greater than or equal to 42,000; it contains the primary  
sequence of seven of the tryptic peptides isolated from the  
insulin-stimulated MAP2 kinase. In addition, the exact  
20 sequences of two additional peptides from a mixed sequence  
are found in the translated cDNA. Together these tryptic  
peptides consisted of 115 residues all of which are in  
accord with sequence in the translated cDNA, accounting for  
over 31% of the putative translation product. The exact  
25 correspondence between the extensive tryptic peptide  
sequence and the predicted translation product provides  
substantial evidence that the cDNA encodes the insulin-  
stimulated MAP2 kinase. We have designated the gene ERK1  
for extracellular signal-regulated kinase-1.

30 ERK1 contains the 15 invariant residues found in all  
protein kinases; it also displays substantial homology with  
all of the subdomains defined by Hanks et al. (1988,  
Science, 241:42-52), and contains residues characteristic  
of serine/threonine protein kinases. A comparison with  
35

other protein kinases reveals striking similarities between MAP2 kinase and the KSS1 (Courchesne, et al., 1989, Cell 58:1107-1119) and FUS3 (Elion, et al., Cell 60:649-664) protein kinases recently isolated from yeast. See *infra* and FIGURE 4. ERK1 kinase is 56% identical to KSS1, and  
5 56% identical to FUS3. KSS1 and FUS3 display about the same degree of similarity (57%) to each other as they do to MAP2 kinase; they are all significantly less homologous to other kinases than they are to each other. All three share their next most impressive homologies with the CDC28/cdc<sup>2+</sup>  
10 subfamily of kinases (ERK1 kinase is 41% identical to this kinase). However, KSS1, FUS3 and MAP2 kinase all lack the VPSTAIR sequence found in subdomain III of all CDC28 functional homologs. Furthermore, all three also share C-terminal extensions not found on the CDC28/cdc<sup>2+</sup> kinases.  
15 Unlike FUS3 and KSS1, MAP2 kinase contains a significant N-terminal extension of at least 17 amino acids.

ERK1 also differs from its yeast homologs at the C-terminus and between the DFG and APE motifs of subdomains VII and VIII, which contain inserts of different lengths  
20 with phosphorylatable residues (e.g. Thr-238 and Thr-242 in ERK1). Both regions poorly conserved between the yeast kinases, have been implicated in determining unique functional characteristics of individual kinases. In a number of kinases the segment that resides between  
25 subdomains VII and VIII is autophosphorylated in a manner that influences enzymatic activity (e.g., cAMP-dependent protein kinases insulin receptor). Conservation among the kinases may also reveal functionally important residues. There is a conserved tyrosine in subdomain 1, whose  
30 phosphorylation in CDC28 is known to inhibit protein kinase activity. Near the C-terminus of the protein is a sequence (residues 367-379) that draws particular attention as a potential site of tyrosine phosphorylation due to interesting similarities to the regulatory  
35

autophosphorylated region of the insulin receptor. Three tyrosine residues are located within this region spaced identically to those in the receptor (YX<sub>3</sub>YY); four acidic residues, important determinants for recognition by protein-tyrosine kinases, are nearby. The presence of  
5 sequences resembling the insulin receptor phosphorylation site is consistent with evidence suggesting that ERK1 (MAP2 kinase) may be a substrate for the insulin receptor.

10 6.2.1. TISSUE DISTRIBUTION AND INDUCIBILITY OF THE MAP2 KINASE TRANSCRIPT

A probe made from the MAP2 kinase cDNA identifies a single 1.9 kb transcript on Northern blot analysis, indicating that our cDNA clone represents nearly full-length transcript. The MAP2 kinase transcript is  
15 detectable in all tissues and cell lines examined. Interestingly, the transcript is expressed at highest levels (by 3- to 6-fold) in the central nervous system compared to other tissues examined. The abundance of MAP2 kinase transcripts in the adult rat brain was estimated to  
20 be about 0.0005% by screening 10<sup>6</sup> phage from a rat brain cDNA library at high stringency. The transcript is clearly detectable in Rat 1 fibroblasts and PC12 cell lines, both of which express acutely activatable MAP2 kinase.

25 6.3. DISCUSSION

Amino acid sequence was obtained from tryptic peptides isolated from a MAP2 protein kinase purified over 6000-fold from insulin-stimulated rat fibroblasts. The sequence was used to design degenerate oligonucleotides that led to the  
30 molecular cloning of a 1.9 kb cDNA via a PCR-based strategy. The cDNA predicts a protein with a molecular weight of greater than or equal to 42,000, designated ERK1, which contains sequences consistent with all of the tryptic peptide sequence obtained from the purified MAP2 kinase,  
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accounting for more than 31% of the primary sequence of the protein. Thus, this cDNA appears to encode the insulin-stimulated MAP2 kinase.

Many protein kinases phosphorylate MAP2 (Aklyama et al., 1986, J. Biol. Chem. 261:14797-14803; Akiyama et al., 1986, J. Biol. Chem. 261:15648-15651; Hernandez et al., 1987, J. Neurochem. 48:84-93). The MAP2 kinase molecularly cloned here is distinguished by its rapid activation by insulin (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719; Ray et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1502-1506; Ray et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:3753-3757; Ray et al., 1988, J. Biol. Chem. 263:12721-12727; Hoshi et al., 1988, J. Biol. Chem. 263:5396-5401; Sturgill et al., 1988, Nature, 334:715-718; Anderson et al., 1990, Nature 343:651-653). Although the physiological functions of this enzyme are not known, a role in the regulation of S6 phosphorylation was proposed following the demonstration that MAP2 kinase phosphorylates and activates S6 kinase II purified from *Xenopus laevis* (Sturgill et al., 1988, Nature 334:715-718) as well as a rabbit liver S6 kinase (Gregory et al., 1989, J. Biol. Chem. 264:18397-18401). The involvement of the MAP2 kinase in signaling pathways is further indicated by the presence of phosphotyrosine on the kinase (Ray et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:3753-3757; Boulton et al., 1991, Biochem. 30:278-286; Boulton et al., May 1991, Cell). While it has not been shown that the insulin receptor catalyzes this phosphorylation, the fact that dephosphorylation of the tyrosine residues decreases activity (Anderson et al., 1990, Nature 343:651-653; Boulton and Cobb, May 1991, in Cell Regulation, Vol. II) supports the notion that the MAP2 kinase functions at an early step in the signal transduction pathway and is, therefore, perhaps directly regulated by the insulin receptor. Additional evidence indicating a role for this

kinase in signaling by numerous agents is raised by evidence indicating that the MAP2 kinase may be pp42 (Rossomando et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6940-6943), a protein whose phosphotyrosine content increases following transformation by viruses and exposure to growth factors (Cooper et al., 1981, Mol. Cell. Biol. 1:165-178). MAP2 kinase, or a closely related enzyme is also activated by a variety of agents that promote differentiation or expression of differentiated functions, not cellular proliferation (Boulton et al., 1990, J. Biol. Chem. 265:2713-2719; Ray et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:1502-1506; Ray et al., 1988, Proc. Nat. Acad. Sci. U.S.A. 85:3753-3757; Ray et al., 1988, J. Biol. Chem. 263:12721-12727; Hoshi et al., 1988, J. Biol. Chem. 263:5396-5401; Ely et al., 1990, J. Cell Biol. 110:731-742; Volonte et al., 1989, J. Cell Biol. 109:2395-2403; Miyasaka et al., 1990, J. Biol. Chem. 265:4730-4735) For example, we have recently purified a nerve growth factor-stimulated MAP2 kinase from PC12 cells to near homogeneity by the same procedure developed to purify the insulin-stimulated enzyme. This enzyme is the same size by SDS-PAGE as the insulin-stimulated kinase. Furthermore, the activity has properties in common with nerve growth factor-activated MAP kinase described by Greene and coworkers (Volonte et al., 1989, J. Cell Biol. 109:2395-2403 and Miyasaka et al., 1990, J. Biol. Chem. 265:4730-4735).

The dramatic homology between ERK1 and the two yeast kinases, KSS1 and FUS3, is consistent with a critical and evolutionarily conserved role for this new family of kinases in mediating the response to extracellular signals. The yeast kinases play antagonistic roles in regulating the yeast cell cycle in response to mating factors, the only known peptide hormones that mediate intercellular communication in yeast. Both kinases seem to act by fine-tuning the activity of CDC28, a related protein-

serine/threonine kinase which is the indispensable regulator of the mitotic cycle, probably via interactions with a yeast cyclin. FUS3 apparently has two regulatory roles. It seems to be important in leading to pheromone-induced cell-cycle arrest in G1, either by directly  
5 inhibiting the activation of CDC28 or by promoting the inactivation of a cyclin required for CDC28 activation; activation of FUS3 by pheromones also independently promotes mating-specific functions. By contrast, KSS1 promotes re-entry into the cell cycle following pheromone-  
10 induced cell-cycle arrest; KSS1 may work by activating the same cyclin that FUS3 may inactivate. ERK1 apparently represents a mammalian counterpart, perhaps functionally as well as evolutionarily, to the yeast kinases. Thus ERK1 may act via similar pathways to regulate the cell cycle in  
15 response to a variety of extracellular signals. As with FUS3, ERK1 apparently also plays a regulatory role in responses that do not directly involve the cell cycle. The dramatic homology between ERK1 and the yeast kinases raises the possibility that yeast may provide a useful  
20 experimental system in which to introduce ERK1 for the analysis of its function.

MAP2 kinase, or MAP2 kinase-like activity, is increased in many different types of cells in response to a wide variety of stimuli. In the course of cloning ERK1, we  
25 have also molecularly cloned other closely related kinases. The identification of a mammalian family of MAP2 kinase-related enzymes, which are structural homologs of the yeast KSS1 and FUS3 kinases, suggests that multicellular, higher eucaryotes have appropriated kinases, originally utilized  
30 to detect environmental perturbation by unicellular organisms, to mediate responses to extracellular signals. The molecular cloning of this family of MAP kinase-related proteins will facilitate the elucidation of the mechanisms

of regulation of this group of enzymes and studies of their physiological roles.

7. EXAMPLE: ERKs, A FAMILY OF PROTEIN-SERINE/-  
THREONINE KINASES THAT ARE ACTIVATED AND  
TYROSINE PHOSPHORYLATED IN RESPONSE TO  
INSULIN AND NGF

5

7.1. MATERIALS AND METHODS

7.1.1. ISOLATION AND SEQUENCE ANALYSIS  
OF NOVEL ERK GENES

The QYDL probe described in Boulton et al. (1990,  
10 Science 249:64-67) was used to screen both a rat brain cDNA  
library constructed in the Lambda Zap II vector  
(Stratagene) as well as a rat genomic DNA library derived  
from rat (Sprague-Dawley) liver DNA partially digested with  
Sau3A restriction endonuclease and then cloned into the  
15 EMBL3/SP6/T7 bacteriophage vector (Clontech). After  
hybridization (Maisonpierre et al., 1990a, Science  
247:1446-1451), the ERK2 and ERK3 phage clones were  
identified (and eventually purified) by washing library  
filters using low-normal stringency (20 mM sodium citrate,  
20 pH 7.0, 0.15 M NaCl, 0.1% sodium dodecyl sulfate (SDS) at  
60°C). The inserts in the phage clones were subcloned into  
Bluescript2 plasmid (Stratagene), and characterized by DNA  
sequence analysis using the dideoxynucleotide chain  
termination method (Sanger et al., 1977, Proc. Natl. Acad.  
25 Sci. U.S.A. 74:5463-6467), with the Sequenase version 2.0  
kit and recommended protocols (U. S. Biochemical).

7.1.2. GENERATION OF ERK-SPECIFIC PROBES

Oligonucleotides (17 bases) corresponding to the DNA  
30 sequences bordering the relatively unconserved N-terminal  
regions of ERK1 (amino acids 5-67), ERK2 (amino acids 14-  
138), and ERK3 (amino acids 11-105), were used to amplify  
precisely these coding regions from plasmids containing  
each of the ERK-cDNA inserts. The N-terminal coding

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regions were used because they were the least homologous regions among these three ERKs. Polymerase chain reaction (PCR) amplifications were also performed, with each pair of these primers, using either rat brain cDNA or genomic rat DNA as template. Each of these amplifications yielded  
5 indistinguishable fragments whether genomic DNA or cDNA was used, indicating that these probes did not span any introns within the ERK genes. The three fragments amplified from plasmids were each radiolabeled using the polymerase chain reaction method and hybridized to Southern blots containing  
10 rat and human genomic DNA (as described in Maisonpierre et al., 1990, Science 247:1446-1451); the filters were hybridized to each of the radiolabeled ERK-specific probes at 68°C in the presence of 0.5 M sodium phosphate, pH 7.0, 1% bovine serum albumin (fraction V, Sigma), 7% SDS, 1 mM  
15 EDTA (Mahmoudi and Lin, 1989, Biotechniques 7:331-333) and 100 µg/ml of sonicated, denatured salmon sperm DNA, and then were washed at 68°C as in Maisonpierre et al. (1990, Science 247:1446-1451) and subjected to autoradiography.

20

#### 7.1.3. NORTHERN BLOT ANALYSIS

Dissections of tissues and brain regions of Sprague-Dawley rats (Harlan Sprague Dawley, Inc.) were performed as described in Maisonpierre et al., 1990, Neuron 5:501-509. The dissected samples were immediately frozen in liquid  
25 nitrogen. Timed-pregnant rats were used to obtain embryonic tissues, with day of sperm positivity designated as day E1; the day of birth was designated P0. Adult rats averaged 150-275 g (6-8 weeks of age). Total RNAs were isolated by homogenization in 3M LiCl/6M urea as described  
30 in Bothwell et al., 1990, "Preparation of DNA and RNA. In Methods For Cloning and Analysis of Eukaryotic Genes", Jones and Bartlett, Boston, MD, pp. 15-16. Gel electrophoresis, capillary transfer to nylon membranes (MagnaGraph, Micro Separations, Inc.), and UV-cross-linking

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to the membranes were performed as described in Maisonpierre et al., 1990, Neuron 5:501-509. The filters were hybridized to the radiolabeled ERK-specific probes and washed as described in the preceding section. Ethidium bromide staining of triplicate gels demonstrated that equivalent amounts of total RNA were being assayed (Maisonpierre et al., 1990, Science 247:1446-1451) on each blot; this was confirmed by hybridizing several of the blots with a probe for 28S rRNA.

10

#### 7.1.4. CULTURING OF ASTRO-GLIAL CELLS AND P19 EMBRYOCARCINOMAS

To obtain purified astroglial cell hippocampi from newborn rats were dissected, dissociated and cultured in serum-containing medium (DMEM supplemented with 10% fetal bovine serum). On culture days 7 and 9, the flasks were shaken vigorously to remove non-astroglial cells. Astroglia were then plated onto 100 mm dishes and cultured for 28 days prior to RNA preparation. The S1801A1 subclone of the P19 embryocarcinoma (McBurney et al., 1982, Nature 299:165-167) was cultured and induced as described in Dinsmore and Solomon, 1991, Cell 64:817-826. RNA was prepared 3 days after induction.

20

#### 7.1.5. BACTERIAL STRAINS AND PLASMIDS

E. coli W3110 lac  $1^{qF-}$  a strain that overproduces the lactose operon repressor, and the plasmid vector pCP110 have been used in studies described in Panayotatos, 1988, Gene 74:357-363. Vectors were engineered for ERK2 expression by using polymerase chain reaction as follows: The 5' synthetic oligodeoxyribonucleotide primer (RAE-21) was designed to generate a unique Sal I site immediately following the initiation methionine ATG codon by changing the sequence GTA-CGA(Val-Arg) into TCG-ACA(Ser-Thr). The 3' primer (RAE22) included a unique Eag I site following

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the TAA termination codon. The expression vector pCP110 was linearized with Sal I plus Eag I and the resulting 3652 bp fragment was purified by agarose gel electrophoresis. The vector and PCR fragment similarly digested and purified were ligated and transformed in E. coli W3110 lac 1<sup>q</sup>F<sup>-</sup>.

5 Transformants were screened by restriction mapping for the desired plasmid and a positive candidate (pRPN117) was confirmed by DNA sequencing to carry the expected full length gene fused to the translation initiation signal in the correct reading frame.

10 pRPN125: This plasmid is identical to pRPN117 except that the codons of the first two amino acids were restored to the native sequence. This was accomplished with two internal PCR primers that extended over the target sequence and carried the desired modifications and two external  
15 primers that served to amplify the desired fragment. Two reactions, each with 1 µg pRPN117 as a template, were set up: one contained 5 µg RAE-10 primer and 0.5 µg RAE-28 primer and the other 5 µg RAE-22 primer and 0.5 µg RAE-27 primer. After ten PCR cycles (each cycle consisting of  
20 incubation for 1 min at 92°C, 2 min at 55°C, 2 min at 72°C) the two samples were combined and subjected to another 25 cycles (consisting of incubation for 1 min at 94°C, 2 min at 55°C, 4 min at 74°C) in the DNA thermal cycler. Because the internal primers RAE-28 and RAE-27 are fully  
25 complementary to each other, the products of the first stage PCR reactions can subsequently anneal. Furthermore, in the second stage reaction, the presence of substantially higher concentrations of the external primers RAE-10 and RAE-22 drives the synthesis of large amounts of the desired  
30 full-length product. The product of the final PCR reaction was purified by PAGE. A 3349 bp fragment was obtained by digesting pRPN117 with Aat II and Eag I and purified by agarose gel electrophoresis. Both fragments were ligated and transformed in E. coli W3110 lac 1<sup>q</sup>F<sup>-</sup>. Transformants  
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were screened by restriction mapping for the desired plasmid and one of the positive candidates (pRPN125) was further characterized by DNA sequencing across the 5' primer region.

5 For fermentation, cells were shaken in LB broth at 37°C to OD<sub>590</sub>=1. Lactose was added to 1% final concentration and incubation continued for 20 hours. Cells were collected by centrifugation at 6,000 x g for 30 min, resuspended in threefold (w/v) excess buffer A (100 mM Tris-HCl pH 7.5, 50 mM EDTA pH 8.0, 0.2 mM DTT) and stored  
10 at -20°C.

#### 7.1.6. PURIFICATION OF RECOMBINANT ERK2

Cells (2 g) were thawed at room temperature, incubated with 2 mg of lysozyme on ice for 20 minutes and passed through a French press (SLM-Aminco) at 8000 psi. The  
15 viscous suspension was then diluted 2-fold with buffer A and further homogenized with 3 1-minute bursts of a Polytron (Kinematica) at a setting of 4. After centrifugation at 11,000 x g for 10 minutes at 4°C, the supernatant was diluted 5-fold with buffer B (20 mM Hepes, pH 7.5, 0.1 mM EDTA, 2 mM dithiothreitol, and 20 mM NaCl)  
20 and applied to Affigel blue (Biorad) equilibrated in buffer B. Proteins were eluted with a 100-ml, linear gradient of 0.02-1 M NaCl in buffer B. Fractions of ERK2 (0.4-1 M NaCl) were diluted with buffer C (20 mM histidine, pH 5.6, 0.1 mM EDTA, 2 mM dithiothreitol, and 20 mM NaCl) to reduce  
25 NaCl to 0.1 M and loaded onto a DEAE-cellulose cartridge (ZetaPrep 60, CUNO) equilibrated in buffer C. The cartridge was washed stepwise with 0.1-1 M NaCl in 0.1 M increments. ERK2 eluted between 0.6 and 0.9 M NaCl and was  
30 judged to be greater than 90% pure (e.g. FIGURE 7C).

#### 7.1.7. MEASUREMENT OF RECOMBINANT ERK2 ACTIVITY

Autophosphorylation of ERK2 was carried out in the presence of 10 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM  
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benzamidine, 30 mM Hepes, pH 8.0, and [ $\gamma$ - $^{32}$ P]ATP at 30°C for 30 minutes. Kinase activity was measured by incubating ERK2 with 50  $\mu$ M ATP, 20 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 1 mM benzamidine and MBP or MAP2 (0.1 mg/ml). The reactions were terminated with 10% trichloroacetic acid as in Boulton et al., 1991, Biochemistry 30:278-286.

7.1.8. LABELING, IMMUNOPRECIPITATION, AND PHOSPHOAMINO ACID ANALYSIS OF ERK1

Rat 1 HIRc B or PC12 cells in 2 100-mm dishes were changed to serum-free Krebs-Ringers-bicarbonate solution in 2% bovine serum albumin (Smith et al., 1980, Proc. Natl. Acad. Sci. U.S.A., 77:2641-2645) for either overnight or 60 minutes and then labeled with  $^{32}$ P orthophosphoric acid (1 mCi/ml) for 50 minutes with or without the addition of insulin (0.18  $\mu$ M) or NGF (75 ng/ml) for the last 5 minutes of the incubation. The cells were washed in chilled medium, scraped in 1 ml of homogenization buffer (20 mM p-nitrophenylphosphate, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA, 50 mM sodium fluoride, 50  $\mu$ M sodium orthovanadate and 5 mM benzamidine) containing 2 mM phenylmethylsulfonyl fluoride and 0.1  $\mu$ M pepstatin, and homogenized by douncing. Following sedimentation at 100,000 x g for 1 hour at 4°C, the supernatants (1 ml) were precleared once with preimmune serum and then divided for incubation with 5  $\mu$ l of either antiserum 837 or preimmune serum for 1 hour on ice. Immune complexes were collected with Pansorbin (Calbiochem) and washed in homogenization buffer plus 0.2% Triton X-100 containing first 2 M NaCl, second 0.15 M NaCl, and finally 0.15 M NaCl plus 0.01% SDS. The pellets were resuspended in 40  $\mu$ l of 2.5-fold concentrated electrophoresis sample buffer, boiled for 10 minutes, and loaded onto a 10% polyacrylamide gel in SDS. Aliquots (5  $\mu$ l) along with an ERK1 standard were transferred to nitrocellulose to confirm by immunoblotting that the radiolabeled band in each

immunoprecipitate comigrated with ERK1. For denaturing immunoprecipitations, the supernatants were adjusted to final concentrations of 0.5% SDS and 1 mM dithiothreitol. The samples were boiled 1-2 minutes and diluted 4-fold with homogenization buffer containing 1.25% sodium deoxycholate, 1.25% Triton X-100, and 0.1 or 1 mM dithiothreitol and then immunoprecipitated as above. The bands corresponding to ERK1 were excised from the dried gels and hydrolyzed in 6 N HCl for 90 minutes. Phosphoamino acids were analyzed as described by Cooper et al., 1983, "Methods In Enzymology," Vol. 99, J. D. Corbin and J. G. Hardman, eds, New York Academic Press, pp. 387-402.

To blot the immune complexes 1 mg of supernatant was immunoprecipitated under denaturing conditions. The Pansorbin pellets were washed twice with 0.25 M Tris, pH 7.5, and 0.1 M NaCl prior to electrophoresis. To detect phosphotyrosine the blot was stained using a monoclonal antibody to phosphotyrosine (UBI) and visualized with a goat anti-mouse IgG alkaline phosphatase-conjugated secondary antibody. The 691 immunoblot was developed with goat anti-rabbit IgG horse radish peroxidase-conjugated secondary antibody.

#### 7.1.9. CHROMATOGRAPHY OF EXTRACTS ON MONO-Q SEPHAROSE

PC12 cells were grown to confluence on uncoated plastic dishes in Dulbecco's Modified Eagles medium with 5% fetal bovine serum and 5% horse serum. NGF was added to 5 of 10 150-mm dishes at a final concentration of 50 ng/ml for 5 minutes. Cells were scraped into homogenization buffer and soluble fractions were prepared and protein determined as described (Boulton et al., 1991, Biochem. 30:278-286). Soluble fractions were diluted with 3 volumes of water and applied to a Mono-Q HR 5/5 column equilibrated in 50 mM  $\beta$ -glycerophosphate, pH 7.3, 1 mM EGTA, 1 mM

dithiothreitol, 0.1 mM sodium vanadate, and 0.1  $\mu$ M pepstatin. The protein was eluted with a gradient of 0-0.3 M NaCl in this buffer. Sixty 1-ml fractions were collected and assayed for MBP kinase activity using 0.3 mg/ml MBP.

5

## 7.2. RESULTS

### 7.2.1. MOLECULAR CLONING OF TWO NOVEL ERKS

The same probe utilized to isolate the ERK1 cDNA (Boulton et al., 1990, Science 249:64-65) was used to screen a rat brain cDNA library at low-normal stringency, resulting in the identification of multiple hybridizing cDNA clones. Analysis of these clones has led to the discovery of at least two novel protein kinases, which we designate ERK2 and ERK3 (FIGURES 3A (SEQ ID NO:3 AND NO:4) and 3B (SEQ ID NO:5 and NO:6)). ERK1, ERK2 and ERK3 are all more closely related to the yeast kinases, KSS1 and FUS3, than to any other protein kinases (FIGURES 4A and 4B). While the ERKs share approximately 37% (ERK3) to 56% (ERKs 1 and 2) identity with the yeast kinases, they are significantly less related (26% identity for ERK3 and 41% identity for ERKs 1 and 2) to their next closest relatives, the cdc2 family of kinases (Lee and Nurse, 1987, Nature 327:31-35). ERK1 and ERK2 are much more closely related to each other (90% identity) than to ERK3 (FIGURE 2B). Using the first in-frame methionine (which satisfies the Kozak consensus for initiation sites (Kozak, 1987, Nucleic Acids Res. 15:8125-8148)), the protein predicted by the ERK2 cDNA has a molecular weight of 41.2 kDa, smaller than ERK1 (~43 kDa), with fewer residues at the amino terminus preceding the catalytic domain. A second in-frame methionine for ERK2 can be aligned with the initiator methionines found in KSS1 and FUS3.

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The deduced amino acid sequence of ERK3 predicts a protein of 62.6 kDa. While the initiator methionine is located just downstream of that predicted for ERK2, ERK3

has a C-terminal extension of approximately 180 amino acids compared to ERK1 and ERK2. Despite this long C-terminal extension, ERK3 is notably more related to ERK1 and ERK2 (~50% identity within the catalytic domain) than to its next closest relatives (FIGURE 4B). Furthermore, clusters  
5 of near identity among the ERKs (e.g. subdomains V-83% identity and VI-95% identity; see FIGURE 4A) demonstrate that ERK1 and ERK2 are more closely related to ERK3 than they are to KSS1 and FUS3. Sequencing of two independent  
10 cDNA clones confirmed that ERK3 contained SPR rather than APE in subdomain VIII; the glutamic acid in the APE sequence is the only one of the fifteen invariant residues in protein kinases (Hanks et al., 1988, Science 241:42-52) which is not conserved in ERK3. Mutational analysis with the src tyrosine kinase has revealed that a lysine residue  
15 substituted for this glutamic acid leads to diminished activity (Bryant and Parsons, 1984, Mol. Cell. Biol. 4:862-866). In subdomain VI, ERK3 contains DLKPAN, a grouping reminiscent of both tyrosine and serine/threonine  
20 kinases.

#### 7.2.2. EVIDENCE FOR ADDITIONAL ERKs

We used probes specific for the individual ERKs (FIGURE 5A) to determine whether or not there are additional members of this family. These probes were  
25 generated from the least conserved portions of each of the three ERKs (see Materials and Methods) and each probe was shown not to cross hybridize to the other known ERKs (FIGURE 5A). The ERK1-specific probe identified a single  
30 strongly hybridizing fragment as well as several weakly hybridizing fragments in rat genomic DNA digested with EcoR1, but multiple strongly hybridizing fragments in rat genomic DNA digested with other enzymes (FIGURES 5B and 5C). The ERK2-specific probe identified two distinct EcoR1  
35 fragments in rat genomic DNA and three distinct EcoR1

fragments in human genomic DNA (FIGURE 5B). The ERK3-specific probe identified two fragments in rat genomic DNA and four fragments in human genomic DNA (FIGURE 5B). Because these probes did not span any introns or contain any of the restriction sites used in the analysis (see  
5 Materials and Methods), these hybridizations suggest that there are multiple ERKs in addition to those already isolated and that the ERKs may be further grouped into subfamilies. Screening rat genomic and cDNA libraries with the ERK-specific probes further supports the idea of  
10 subfamily members due to the isolation of a number of clones that hybridize only to one of the specific probes, but only at low-normal stringency. However, these may not all represent functional genes because partial nucleotide sequence of one of the genomic clones reveals that it  
15 contains a pseudogene closely related to ERK1 (designated ERK1 $\psi$ , FIGURE 3C).

#### 7.2.3. DISTINCT DEVELOPMENTAL AND TISSUE DISTRIBUTIONS OF ERKs

20 The ERK1-, ERK2-, and ERK-3 specific probes were used to determine the developmental and tissue distributions of ERK mRNA expression. In the adult rat all three ERK mRNAs were expressed at highest levels within the nervous system, although all the mRNAs were detectable in all tissues  
25 examined (FIGURE 6A). Within the nervous system ERK2 and ERK3 displayed a clearly reciprocal pattern of mRNA expression, with higher ERK3 expression in hindbrain regions and higher ERK2 expression in forebrain regions; by comparison ERK1 was expressed more uniformly. Outside of  
30 the nervous system, each of the ERKs was expressed at highest amounts in different tissues. ERK1 was expressed at highest levels in intestine and placenta and to a lesser extent in lung. ERK2 mRNA was expressed at highest levels in muscle, thymus, and heart. The ERK2 probe identified

three distinct transcripts, which were expressed at different ratios in different tissues; these transcripts may be differentially processed forms of the ERK2 mRNA or may arise from other genes in the ERK2 subfamily. ERK3 mRNA was expressed at highest levels in skeletal muscle.

5       A developmental study of the expression of ERK1, ERK2, and ERK3 within the nervous system revealed that ERK3 mRNA was expressed at highest levels early in development (especially in spinal cord and hippocampus), while the expression of ERK1 and ERK2 mRNAs generally increased  
10       during development (FIGURE 6B). The developmental increases in the mRNAs for two of these kinases within the brain reflected changes in the amounts of ERK1 and ERK2 protein (see below). In liver and heart the expression of all three ERKs decreases in the adult rat (FIGURE 6B). The  
15       discrete distributions and developmental patterns of the ERKs suggest that they play unique physiological roles in different cells or perhaps in response to different repertoires of signals.

20       The low levels of ERK2 and ERK3 transcripts in sciatic nerve (which contains neuronal axons and neuron-supporting cells (glia), but lacks neuronal cell bodies and thus neuronal-specific mRNA) contrasts with the high levels of ERK1 mRNA in this peripheral nerve (FIGURE 6A). Thus, the high levels of ERK2 and ERK3 in the brain as opposed to  
25       peripheral nerve might reflect specific expression within neurons. To further explore this possibility, the level of each of the ERKs was compared in whole brain and in neuron-free glial cultures derived from newborn brains (FIGURE 6C). The decreased level of ERK2 and ERK3  
30       expression compared to ERK1 expression in these cultures further indicates that neuronal specificity of ERK2 and ERK3.

7.2.4. DISTINCT REGULATION OF ERK TRANSCRIPTS  
UPON INDUCTION OF NEURONAL OR MUSCLE  
DIFFERENTIATION IN EMBRYOCARCINOMA CELLS

The embryonal carcinoma line P19 normally displays an undifferentiated phenotype, but can be induced to differentiate into neuronal muscle lineages following treatment with retinoic acid (RA) or dimethylsulfoxide (DMSO), respectively (McBurney et al., 1982, Nature 299:165-167). Transcripts hybridizing to the three ERK probes are each uniquely regulated during P19 differentiation. While ERK1 transcripts display no or a slight decrease upon induction towards either neuronal or muscle-like phenotypes, the ERK2 transcripts display a notable increase only upon neuronal induction (which parallels the pattern seen for the low affinity NGF receptor) and the ERK3 transcripts increase upon differentiation toward either the neuronal or muscle lineage. These expression patterns upon differentiation parallel the specific distributions each of the ERKs display in vivo (see above), and provide additional support for the neuronal (rather than glial) specificity of both ERK2 and ERK3. Thus, the P19 differentiation system appears to be a useful model system for studying the roles of individual ERKs during neuronal and muscle differentiation. Interestingly MAP2, which may represent a normal substrate for at least some of the ERKs, is apparently required for important features of the P19 neuronal differentiation process such as neurite extension and cessation of proliferation (Dinsmore and Solomon, 1991, Cell 64:817-826).

30

7.2.5. ACTIVITY OF RECOMBINANT ERK2

To begin to compare the properties of the novel ERKs with those of ERK1 purified from insulin-treated fibroblasts (Boulton et al., 1991, Biochem. 30:278-286), we

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purified recombinant ERK2 synthesized in E. coli (FIGURE 7A). Purified recombinant ERK2 phosphorylated itself (FIGURE 7C) and the exogenous substrates MAP2 and MBP in a time- (FIGURE 7D) and concentration-dependent manner. The specific activities of two preparations of purified  
5 recombinant ERK2 were 0.6 and 1 nmol/min/mg of protein with either MAP2 or MBP as substrates. This compares to a specific activity with MAP2 of 300 nmol/min/mg for a highly purified preparation of ERK1 and 4 nmol/min/mg for the same ERK1 preparation which had been exhaustively  
10 dephosphorylated with the catalytic subunit of phosphatase 2a. The fact that recombinant ERK2 has a specific activity similar to that of dephosphorylated native ERK1 suggests that the recombinant protein is in the appropriate conformation to be activated by phosphorylation.  
15

#### 7.2.6. ANTISERA CAN DISTINGUISH ERK1 AND ERK2 AND IDENTIFY A NOVEL ERK

Two different polyclonal antisera, both raised against a peptide consisting of the 16 C-terminal residues of ERK1  
20 (of which 10 are conserved in ERK2), were able to distinguish between purified ERK1 and recombinant ERK2 on immunoblots and could also identify these proteins as well as a novel ERK in crude cell extracts. Antiserum 837 recognized both purified ERK1 and recombinant ERK2 (FIGURE  
25 7B), and also identified two proteins of similar sizes in crude brain extracts (FIGURE 8C). Antiserum 956 recognized purified ERK1 but not recombinant ERK2; in crude brain extracts this antiserum recognized a protein comigrating with purified ERK1 as well as a novel ERK of 45 kDa (FIGURE  
30 8B). Antibodies raised against peptides from other subdomains of ERK1 also recognized all three of these proteins (Boulton and Cobb, May 1991, in Cell Regulation, Vol. II) verifying the identification of ERK1 and ERK2 and confirming the existence of a novel 45 kDa ERK. By  
35



immunoblotting, the amounts of both the 43 and the 41 kDa proteins increased dramatically (FIGURES 8B and 8C) in adult brain compared to embryonic brain, paralleling the developmentally regulated increase in accumulation of mRNAs for ERK1 and ERK2 in brain. Based on the specificities of the antisera for the purified ERKs as well as the correlations between amounts of protein and mRNA, we have designated the 43 kDa protein recognized in crude brain extracts by both antisera as ERK1, the 41 kDa protein recognized only by antiserum 837 as ERK2, and the novel 45 kDa ERK as ERK4.

#### 7.2.7. ERKS ARE PHOSPHORYLATED IN RESPONSE TO INSULIN AND NGF

It has been reported that a protein cochromatographing with MAP kinase activity was phosphorylated on threonine and tyrosine residues in response to insulin (Ray and Sturgill, 1988, Proc. Natl. Acad. Sci. U.S.A. 85:3753-3757). Both types of phosphorylation may be required for maximum MAP2 kinase activity (Anderson et al., 1990, Nature 343:651-653; Ahn et al., 1990, J. Biol. Chem. 265:11495-11591; Gomez et al., 1990, FEBS Lett. 271:119-122; Boulton and Cobb, 1991, Cell Regulation, In press). We utilized our antibody reagents that specifically identify individual ERKs to explore the activation and phosphorylation state of individual members of this kinase family in response to two different growth factors, insulin and NGF.

To examine the effect of insulin and NGF on ERK1 phosphorylation directly, we used antiserum 837 to immunoprecipitate ERK1 from <sup>32</sup>P-labeled Rat 1 HIRcB or PC126 cells before and after insulin or NGF stimulation (FIGURE 9A). Although antiserum 837 recognized ERK1, ERK2, and ERK4 on immunoblots under denaturing conditions, it immunoprecipitated the 43 kDa ERK1, a small amount of the 45 kDa ERK4, but not the 41 kDa ERK2 from crude extracts

under nondenaturing conditions. No  $^{32}\text{P}$ -labeled bands were detected in immunoprecipitates from untreated cells, while  $^{32}\text{P}$ -labeled ERK1 (43 kDa) and a small amount of  $^{32}\text{P}$ -labeled ERK4 (45 kDa) were immunoprecipitated from both insulin-treated or NGF-treated cells, indicating that there is hormone-dependent phosphorylation of both of these ERKs (FIGURE 9A). Under denaturing conditions small amounts of labeled ERK2 were also detectably precipitated following stimulation by NGF (FIGURE 9B) indicating that ERK2, like ERK1 and ERK4, underwent hormone-dependent phosphorylation.

To determine if these phosphorylations include tyrosine, both phosphoamino acid analysis and phosphotyrosine immunoblotting were performed on immunoprecipitated ERKs. When duplicate immunoprecipitates (performed on Rat 1 HIRCB cells under denaturing conditions (see Methods)) were immunoblotted with ERK antibodies as well as antibodies to phosphotyrosine, it was evident that both ERK1 and ERK2 from insulin-stimulated extracts contained phosphotyrosine, while there was little detectable phosphotyrosine on the proteins from the unstimulated cells (FIGURE 10). No phosphotyrosine was detected on the ERK4; this could be due to our inability to detect it with the antibodies because so little of this protein was immunoprecipitated; alternatively, the hormone-induced increase in phosphate on ERK4 may be only on serine/threonine residues. Phosphoamino acid analysis of  $^{32}\text{P}$ -labeled 43 kDa ERK1 excised from the gel of FIGURE 9A revealed that threonine, serine, and tyrosine were phosphorylated in response to NGF (FIGURE 9C). The same was true for ERK1 from insulin-treated cells. These findings demonstrate that at least three ERK proteins are phosphorylated in response to insulin and NGF and at least two of these contain phosphotyrosine.

#### 7.2.8. RELATIONSHIP BETWEEN PHOSPHORYLATION AND ACTIVATION OF ERKS IN RESPONSE TO NGF

To relate the phosphorylation of these proteins to their activities following NGF stimulation, we located ERK1 and ERK2 on immunoblots from Mono Q profiles of untreated and NGF-treated PC12 cells using antisera 956 and 837. NGF treatment resulted in a shift of elution of a portion of both ERK1 (from fractions 29-31 to fractions 38-41) and apparently also ERK2 (from fractions 25-27 to fractions 29-31) (FIGURE 11), presumably due to changes in the phosphorylation of these proteins. Identical immunoblots probed with antiphosphotyrosine antibodies revealed little to no phosphotyrosine on ERK1 or ERK2 before NGF treatment. NGF treatment resulted in increased phosphorylation of tyrosine residues on both ERK1 and ERK2. Phosphotyrosine was detectable in both unshifted and shifted ERK1 protein, suggesting that multiple modifications (such as further phosphorylations on tyrosine, threonine, and/or serine residues, which would be consistent with the phosphoamino acid analysis of immunoprecipitated ERK1 described above) are required to retard elution from Mono Q. Analysis of bands from insulin-treated cells on the same gel indicated that the ERK-crossreacting and phosphotyrosine-containing bands comigrate.

NGF treatment also resulted in two major peaks of MBP kinase activity in the Mono Q profile that were not present in the profile from untreated cells. The first peak of activity coeluted with the shifted ERK2 protein (but also overlapped with the unshifted ERK1 protein), while the second peak coeluted with the shifted ERK1 protein. The first peak of MBP kinase activity was not immunoprecipitable with antiserum 837, unlike the second peak. Further, some activity from the first peak could be precipitated with an antibody to recombinant ERK2 that has a limited ability to immunoprecipitate ERK2, but none could

be precipitated from the second peak. In addition, activity from both peaks could be inactivated by both the serine/threonine-selective phosphatase 2a and by the tyrosine-selective phosphatase CD45 (Boulton and Cobb, May 1991, in Cell Regulation, Vol. II). Thus, these  
5 observations support the conclusion that activity in the second peak is due to fully modified ERK1 and that in the first peak is due to activated ERK2 and not to partially modified ERK1. Altogether, the data indicate that ERK1 and ERK2 are rapidly activated in response to extracellular  
10 signals such as insulin and NGF, and that this activation is correlated with increased tyrosine phosphorylation, but that full activation requires additional modifications.

### 7.3. DISCUSSION

15 We have compared the sequences of three members of the ERK family. Hybridizations to genomic rat and human DNA (which define a minimum of 3 new ERK genes in addition to ERKs 1, 2, and 3 in rat), screening of genomic and cDNA libraries, and immunoblotting with ERK specific antisera  
20 (which define at least one novel ERK in addition to ERK2 and ERK3) suggest that there are additional ERK genes in rat and perhaps even more in human. There is evidence for stimulation of MAP2/MBP Kinase activity in different cell types in response to a variety of signals; there is even  
25 more extensive evidence for tyrosine phosphorylation of 40-45 kDa proteins in response to cellular stimulation (Cooper and Hunter, 1981, Mol. Cell. Biol. 1:165-178; Maytin et al., 1984, J. Biol. Chem. 259:12135-12143; Cooper et al., 1984, Mol. Cell. Biol. 4:30-37; Martinez et al.,  
30 1982, Mol. Cell. Biol. 2:653-685; Cooper et al, 1982, Cell 31:263-273; Rossomando et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6940-6943; Ferrell and Martin, 1990, Molec. Cell. Biol. 10:3020-3025; Gold et al., 1990, Nature 345:810-813). Our results indicate that the MAP2/MBP Kinase activity  
35

measured in extracts from insulin- and NGF-stimulated cells is derived not from a single enzyme but from at least two different protein kinases, ERK1 and ERK2. These data are consistent with our findings that suggest an extended family of ERK-related enzymes in rat. We conclude that  
5 similar activities found in other types of cells in response to other stimuli could be due to ERK1 or ERK2 alone, to both enzymes, or perhaps to other members of this family. The first three characterized members of this family were isolated from a brain cDNA library and are  
10 found at highest levels within the nervous system, although the expression of each ERK displays distinct tissue and developmental regulation. Screening of libraries from other tissues may yield ERKs that function predominantly in non-neural tissues.

15 The kinases next most closely related to the ERKs are the products of the KSS1 (Courchesne et al., 1989, Cell 58:1107-111) and the FUS3 (Ellon et al., 1990, Cell 60:649-664) genes previously cloned from yeast. KSS1 overcomes mating factor-induced growth arrest. In contrast  
20 FUS3 leads to the arrest of the cell cycle in response to mating factors. Because of regions of new identity among all three ERKs, it seems likely that they evolved from a common precursor. The relationship between the ERKs and their yeast relatives suggests that multicellular higher  
25 eukaryotes have appropriated kinases, originally utilized by primordial mating responses in unicellular organisms, to mediate responses to extracellular signals. Furthermore, the cdc2 kinases, which are the next closest relatives of the ERKs, apparently play similar roles in regulating the  
30 cell cycle in eukaryotes as diverse as yeast and man. Intriguingly, as in the case with the ERKs, the cdc2 kinases are also regulated (albeit negatively) by phosphorylation on both tyrosine and threonine.

Understanding signal transduction requires elucidating the mechanisms by which receptor-activated tyrosine phosphorylation is converted into the serine/threonine phosphorylations that regulate downstream targets. We have defined a family of serine-threonine protein kinases, the ERKs, with members that are activated and phosphorylated on tyrosine residues in response to NGF and insulin. The initiation of the series of events culminating in activation of the ERKs by insulin and NGF may occur via distinct receptors; however, both hormones are known to elicit tyrosine phosphorylation. While the insulin receptor has been recognized to contain intrinsic tyrosine kinase activity for several years, it is still unclear how activation of the NGF receptor elicits intracellular tyrosine phosphorylation. Recent evidence suggests that the NGF receptor may either contain tyrosine kinase activity (Kaplan et al., 1991, *Nature* 350:158-160) or is associated with such protein (Meakin and Shooter, 1991, *Neuron* 6:153-163). Whatever mechanisms may be involved, phosphorylation of the ERKs represents the first example of defined intracellular proteins which are phosphorylated on tyrosine in response to NGF.

Our findings suggest that a characteristic property of this family of kinases is to serve as intermediates that depend on tyrosine phosphorylation to activate serine/threonine phosphorylation cascades in response to a wide variety of extracellular signals, although it is still unclear whether the ERKs are direct substrates for receptor-associated tyrosine kinases, or whether they are further downstream in cascades. Delineating the involvement of individual ERKs in phosphorylation networks, and how they might act combinatorially, may be necessary to determine how different cell types produce the complex array of responses to the many extracellular signals that activate tyrosine phosphorylation. For example, the same

signal acting through the same receptor, during different developmental stages or in different cell types, can generate dissimilar responses (e.g., proliferation vs. differentiation). Activation of specific ERKs in different contexts may contribute to multiple interpretations of the same signal or common responses to different signals. The observation that the three kinases are differentially expressed in tissues during development and the finding that ERKs 2 and 3 are induced in a model system for neuron development while ERK1 is lost during this process, further support the notion that these enzymes play unique roles in signal transduction pathways recruited during development. Furthermore, deregulation of such potentially important signalling molecules might be involved in cellular transformation and oncogenesis.

The cloning of the ERK genes and the identification of a differentiating cell line in which they are independently regulated may be used in elucidating the role of the ERKs in phosphorylation cascades, and to reveal the mechanisms involved in regulating this family of kinases. Expression of recombinant ERK proteins and their mutants may be used to define the roles of these kinases in vivo and to determine residues involved in ERK function and activation. Antibodies to common and unique regions of the ERKs may facilitate examination of how individual members participate in various responses. The indication from Mono Q profiles that both active and inactive ERK1 contains phosphotyrosine upon stimulation suggests that the extent of ERK1 activation is also determined by threonine/serine phosphorylation. The large increase in MBP kinase activity induced by okadaic acid (Haystead et al., 1990, J. Biol. Chem. 265:16571-16580), is consistent with this notion. The availability of recombinant proteins may be used to dissect the roles of both serine/threonine and tyrosine phosphorylation in ERK activation. In this regard,

recombinant ERK2 has activity similar to dephosphorylated native ERK1, suggesting that the recombinant protein may be in the appropriate conformation to be activated by phosphorylation. In fact, experiments show that the activity of dephosphorylated ERK1 is increased to the  
5 specific activity of the purified active protein and the activity of recombinant ERK2 is increased 150-to 200-fold by an EGF-sensitive activator recently described by Ahn et al., 1991, J. Biol. Chem. 266:4220-4227). The ability to  
10 activate recombinant ERK2 in vitro verifies the utility of recombinant ERK proteins to search for shared or unique substrates, activators (e.g. serine/threonine and tyrosine kinases) and inactivators (e.g., phosphatases).

#### 8. DEPOSIT OF MICROORGANISMS

15 Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

The following plasmids and cell line were deposited with the American Type Culture Collection in Rockville, Maryland.

|    |                        |                      |                        |
|----|------------------------|----------------------|------------------------|
| 20 |                        | <u>Accession No.</u> | <u>Date of Deposit</u> |
|    | Cell line Rat 1 HIRc B | CRL 10476            | May 31, 1990           |
|    | Plasmid pBS-rERK1      | 40808                | May 23, 1990           |
|    | Plasmid pBS-rERK2      | 40809                | May 23, 1990           |
|    | Plasmid pBS-rERK3      | 75009                | May 14, 1991           |

25

The present invention is not to be limited in scope by the constructs deposited or the embodiments disclosed in the examples, which are intended as illustrations of a few aspects of the invention and any embodiments which are  
30 functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art and are intended to fall within the scope of the appended claims.

35



Various references are cited herein, the disclosures of which are hereby incorporated by reference in their entireties.

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International Application No: PCT/

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## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 66, line 15 of the description \*

## A. IDENTIFICATION OF DEPOSIT \*

Further deposits are identified on an additional sheet ☒ \*

Name of depository institution \*

American Type Culture Collection

Address of depository institution (including postal code and country) \*

12301 Parklawn Drive  
Rockville, MD 20852

Date of deposit \*

May 31, 1990

Accession Number \*

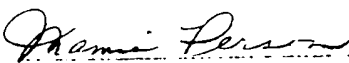
CRL 10476

B. ADDITIONAL INDICATIONS \* (leave blank if not applicable). This information is continued on a separate attached sheet ☒

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE \* (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS \* (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later \* (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)  
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau is \*

was

(Authorized Officer)

PCT/RO/134 (cont'd)

American Type Culture Collection  
12301 Parklawn Drive  
Rockville, MD 20852

Date of deposit: May 23, 1990

Accession Number: 40808

Date of deposit: May 23, 1990

Accession Number: 40809

Date of deposit: May 14, 1991

Accession Number: 75009

WHAT IS CLAIMED IS:

1. An isolated recombinant nucleic acid molecule encoding a mammalian MAP2 kinase.
2. The recombinant nucleic acid molecule of claim 1  
5 comprising a nucleic acid sequence substantially as depicted in Figure 2B (SEQ ID NO:1).
3. The recombinant nucleic acid molecule of claim 2  
10 as comprised in the vector pBS-ERK1, deposited with the ATCC and having accession number 40808.
4. The recombinant nucleic acid molecule of claim 1  
which encodes a serine-threonine protein kinase, having a  
molecular weight between about 43 and 48 kilodaltons.
- 15 5. An isolated recombinant nucleic acid molecule comprising a mammalian nucleic acid which is capable of hybridizing to a nucleic acid molecule having a nucleic acid sequence substantially as depicted in Figure 2B (SEQ  
20 ID NO:1), or a portion thereof.
6. An isolated recombinant nucleic acid molecule which encodes a protein having the amino acid sequence substantially as depicted in Figure 2B (SEQ ID NO:2), or a  
25 portion consisting of at least six amino acids thereof.
7. An organism containing the recombinant nucleic acid molecule of claim 1 or 6.
- 30 8. A cell containing the recombinant nucleic acid molecule of claim 1, 2 or 6.
9. The cell of claim 8 which is derived from the nervous system.

10. The recombinant nucleic acid molecule of claim 1 or 6, which further comprises a nucleic acid sequence capable of controlling gene expression.

11. A substantially purified MAP2 protein kinase molecule comprising the amino acid sequence substantially as depicted in Figure 2B (SEQ ID NO:2), or a portion consisting of at least six amino acids thereof.

12. The substantially purified MAP2 protein kinase of claim 11 which has a molecular weight of between about 41 and 48 kilodaltons as measured by SDS polyacrylamide gel electrophoresis.

13. A substantially purified MAP2 protein kinase molecule comprising (a) a portion homologous to the yeast FUS3 or KSS1 protein kinase and, (b) a nonhomologous amino terminal portion of about 69 amino acids.

14. A substantially purified MAP2 protein kinase encoded by the recombinant nucleic acid molecule of claim 3 or 5.

15. A six amino acid portion of the recombinant MAP2 protein kinase of claim 11.

16. A monoclonal antibody which recognizes the recombinant MAP2 protein kinase of claim 11.

17. An isolated recombinant nucleic acid molecule comprising a nucleic acid sequence substantially as depicted in Figure 3A (SEQ ID NO:3).

18. The recombinant nucleic acid molecule of claim 17 as comprised in the vector pBS-rERK2, deposited with the ATCC and having accession number 40809.

19. The recombinant nucleic acid molecule of claim 17 which encodes a serine-threonine protein kinase, having a molecular weight between about 41 and 48 kilodaltons.

20. An isolated recombinant nucleic acid molecule comprising a mammalian nucleic acid which is capable of hybridizing to a nucleic acid molecule having a nucleic acid sequence substantially as depicted in Figure 3A (SEQ ID NO:3), or a portion thereof.

21. An isolated recombinant nucleic acid molecule which encodes a protein having the amino acid sequence substantially as depicted in Figure 3A (SEQ ID NO:4), or a portion consisting of at least six amino acids thereof.

22. An organism containing the recombinant nucleic acid molecule of claim 21.

23. A cell containing the recombinant nucleic acid molecule of claim 17 or 21.

24. A substantially purified MAP2 protein kinase molecule comprising the amino acid sequence substantially as depicted in Figure 3A (SEQ ID NO:4), or a portion consisting of at least six amino acids thereof.

25. The substantially purified MAP2 protein kinase of claim 24 which has a molecular weight of between about 41 and 48 kilodaltons as measured by SDS polyacrylamide gel electrophoresis.

26. A substantially purified MAP2 protein kinase encoded by the recombinant nucleic acid molecule of claim 18 or 20.

27. A six amino acid portion of the recombinant MAP2 protein kinase of claim 21.

28. The recombinant nucleic acid molecule of claim 1 comprising a nucleic acid sequence substantially as depicted in Figure 3B (SEQ ID NO:5).

5

29. The recombinant nucleic acid molecule of claim 28 as comprised in the vector pBS-rERK3, deposited with the ATCC and having accession number 75009.

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30. An isolated recombinant nucleic acid molecule comprising a mammalian nucleic acid which is capable of hybridizing to a nucleic acid molecule having a nucleic acid sequence substantially as depicted in Figure 3B (SEQ ID NO:5), or a portion thereof.

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31. An isolated recombinant nucleic acid molecule which encodes a protein having the amino acid sequence substantially as depicted in Figure 3B (SEQ ID NO:6), or a portion consisting of at least six amino acids thereof.

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32. An organism containing the recombinant nucleic acid molecule of claim 28.

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33. A cell containing the recombinant nucleic acid molecule of claim 28 or 31.

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34. The recombinant nucleic acid molecule of claim 31 which further comprises a nucleic acid sequence capable of controlling gene expression.

35

35. A substantially purified MAP2 protein kinase molecule comprising the amino acid sequence substantially as depicted in Figure 3B (SEQ ID NO:6), or a portion consisting of at least six amino acids thereof.

36. A substantially purified mammalian protein kinase homologous to the recombinant MAP2 protein kinase of claim 35.

37. A substantially purified MAP2 protein kinase encoded by the recombinant nucleic acid molecule of claim 5 29 or 34.

38. A method of detecting the presence of a compound having nerve growth factor-like activity, comprising:

- 10 (i) culturing cells that produce a MAP2 protein kinase that is activated by nerve growth factor in the presence of a compound suspected of having nerve growth factor-like activity; and
- 15 (ii) detecting changes in the level of the MAP2 protein kinase activity,
- wherein an increase in activity is indicative of the presence of nerve growth factor-like activity.

39. The method of claim 38 in which the cells are 20 PC12 cells.

40. The method of claim 38 in which the cells contain a recombinant nucleic acid molecule encoding a mammalian MAP2 kinase.

25

41. A method of detecting the presence of a compound having insulin-like activity, comprising:

- 30 (i) culturing cells that produce a MAP2 protein kinase that is activated by insulin in the presence of a compound suspected of having insulin-like activity; and
- (ii) detecting changes in the level of the MAP2 protein kinase activity,

35



wherein an increase in activity is indicative of the presence of insulin-like activity.

42. The method of claim 41 in which the cells are Rat 1 HIRc B cells.

5           43. The method of claim 41 in which the cells contain a recombinant nucleic acid molecule encoding a mammalian MAP2 kinase.

10           44. A method of detecting the presence of a compound which directly or indirectly causes a change in the levels of a MAP2 protein kinase activity, comprising:

- (i) culturing cells that produce a MAP2 protein kinase in the presence of a compound; and
- (ii) detecting changes in the level of the MAP2 protein kinase activity,

15           wherein a change in activity is indicative of the presence of the compound.

20           45. The method of claim 43 in which the compound is a neurotrophin molecule, and the change in activity is an increase in activity.

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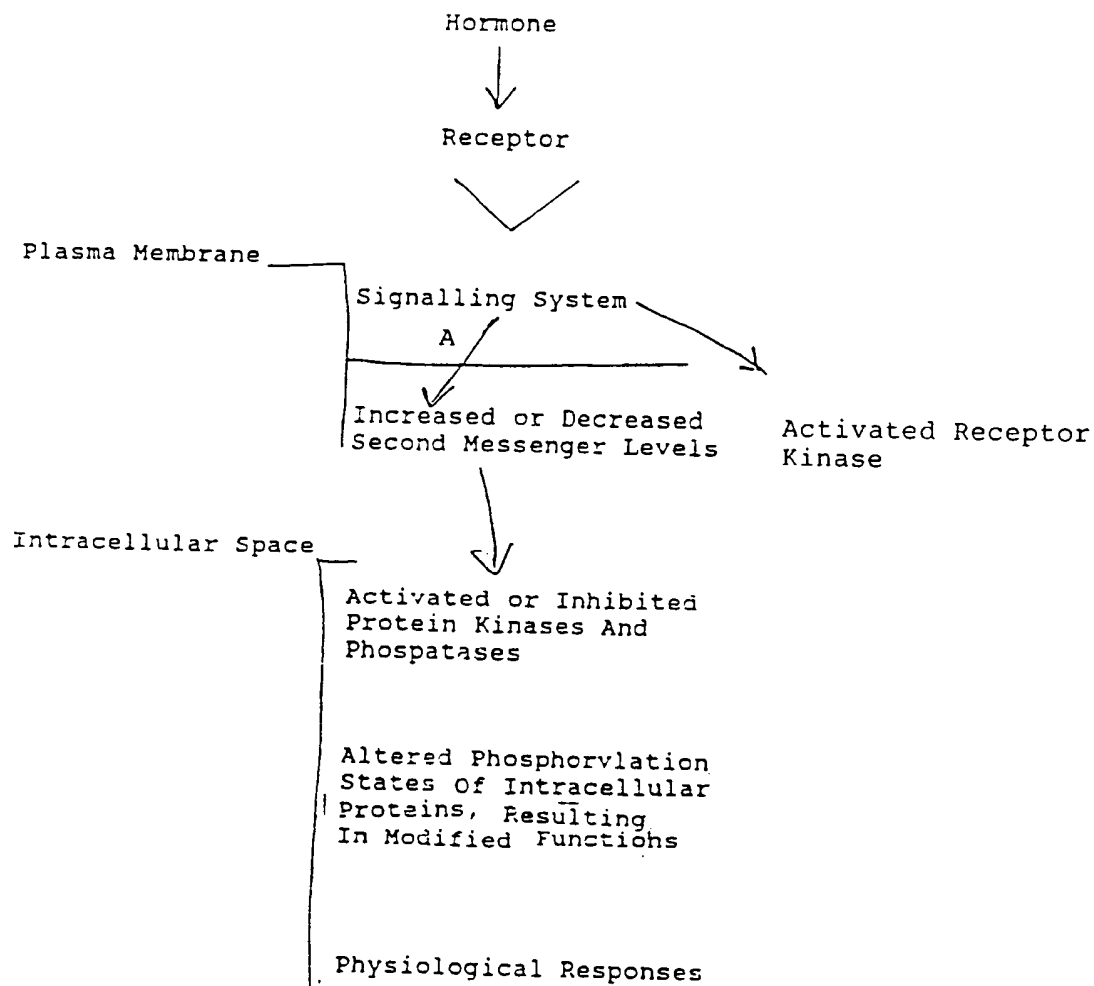
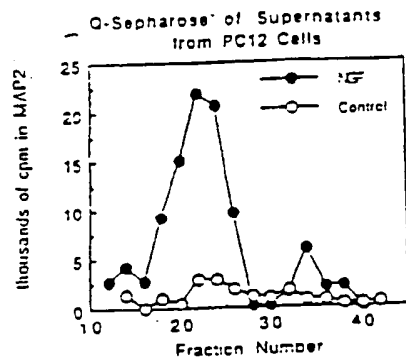


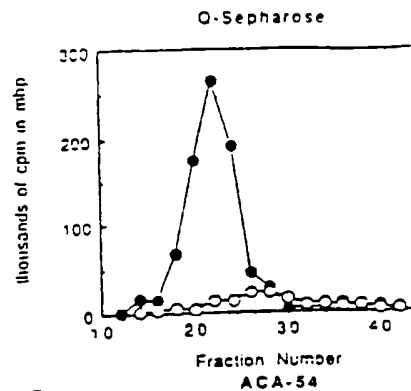
Figure 1

FIGURE 2A

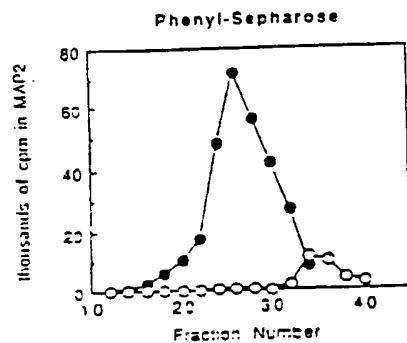
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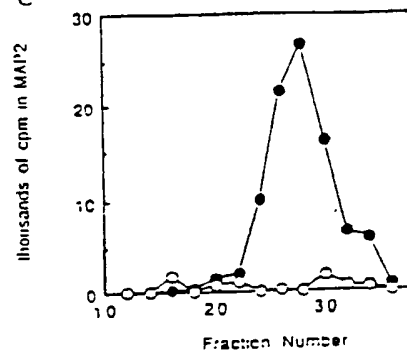
A2



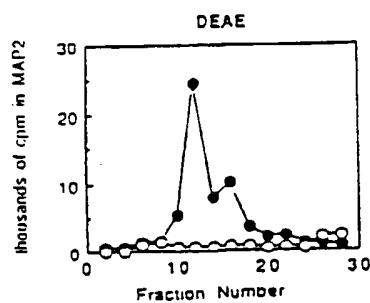
B



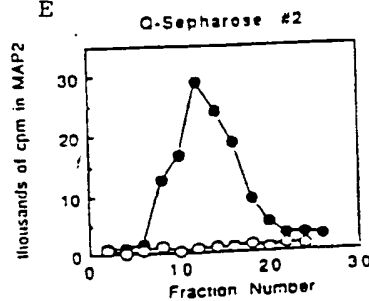
C



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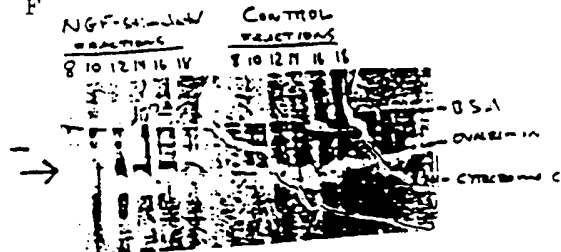


Figure 2 B.

[illegible]

Pro Ttp Phe Tyr Arg Arg Val Leu Ser Ser Val Leu Ser Val Leu Val Pro Asp Arg Arg Gln Pro Ala Thr Arg Ala Ala Arg Phe Leu Trp Glu Ala Gln  
CCG TGG TTC TAC CGG TTA GTT CTC TCT TCT GTG TTG TCC TCC CTC GTT CCC GAT CGC CGC CAG CGC GCT ACA CGC GCG CGC GCG TTC CTG TCG GAA CGC CAG 111

Hls Lys Ser Ser Gly Asn Ala Lys Arg Arg Ala Gln Arg Gly Gly Cys Ala Ala Asn MET Ala Ala Ala Ala Ala Gln Gly Pro Glu MBT Val Arg Gly Gln Val Phe  
CAC MAG TCG AGC GGT AAC CGC MAG CGT CCA GCC CAA CGC CGC GGA GGC TGT GCA GCC AAC ATG CGC GCG GCG GCG GCG CGC CGC GAG ATC GTC CGC GAG CTG TTC 222

Asp Val Gly Pro Arg Tyr Thr Asn Leu Ser Tyr Ile Gly Glu Gly Ala Tyr Gly Met Val Cys Ser Ala Tyr Asp Asn Leu Asn Lys Val Arg Val Ala Ile Lys Lys Ile  
GAC GTG GGG CGC CGC TAC ACT AAT CTC TCG TAC ATC GGA GAA GGC GGC TAC GGC ATG GTT TGT TCT GCT TAT GAT AAT CTC AAC AAA GTT CCA GTT AAT CCA AAA ATC 333

Ser Pro Phe Glu His Gln Thr Tyr Cys Gln Arg Thr Leu Arg Arg Thr Leu Lys Ile Lys Ile Leu Arg Phe Arg His Glu Asn Ile Ile Gly Ile Asn Asp Ile Ile Arg Ala Pro  
AGT CCT TTT GAG CAC CAG ACC TAC TGT CAG AGA ACC CTG AGA GAG ATA AAA ATC CTA CTG CGC TTC AGA CAT GAG AAC ATC ATC GGC AAT GAC ATC ATC CGC GCA CCA 444

Thr Ile Glu Gln Met Lys Asp Val Tyr Ile Val Gln Asp Leu Met Glu Thr Asp Leu Tyr Lys Leu Leu Lys Thr Gln His Leu Ser Asn Asp His Ile Cys Tyr Phe Leu  
ACC ATT GAG CAG ATG AAA GAT GTA TAT ATA GTA CAG GAC CTC ATG GAG ACA GAT CTT TAC AAG CTC TTG AAG ACA CAG CAC CTC AGC AAT GAT CAT ATC TGC TAT TTT CTT 555

Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asn Val Leu His Arg Asp Leu Lys Pro Ser Asn Leu Leu Leu Asn Thr Thr Cys Asp Leu Lys Ile Cys Asp  
TAT CAG ATC CTG ACA GGA TTA AAG TAT ATA CAT TCA GCT AAT GTT CTG CAC CGT GAC CTC AAG CCG TCC AAC CTC CTG CAG AAC ACC ACT TGT GAT CTC AAG ATC TGT GAC 666

Phe Gly Leu Ala Arg Val Ala Asp Pro Asp His Asp His Thr Gly Phe Leu Thr Glu Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met Leu Asn Ser Lys Gly  
ITT GGC CTT GCC CGT GTT GCA GAT CCA GAC CAT GAT CAT ACA GGG TTC TTG ACA GAG TAT GTA GCC AGC CGT TGG TAC AGA GCT CCA GAA ATT ATG TTG AAT TCC AAG GGT 777

Tyr Thr Lys Ser Ile Asp Ile Trp Ser Val Gly Cys Ile Leu Ala Glu Met Leu Ser Asn Arg Pro Ile Phe Pro Gly Lys His Tyr Leu Asp Gln Leu Asn His Ile Leu  
TAT ACC AAG TCC ATT GAT ATT TGG TCT CTG GGC TGC ATC CTG CCA GAG ATG CTA TCC AAC AGC CCT ATC TTC CCA GGA AAG CAT TAC CTT GAC CAG CTG AAT CAC ATC CTC 888

Gly Ile Leu Gly Ser Pro Ser Gln Glu Asp Leu Asn Cys Ile Ile Asn Leu Lys Ala Arg Asn Tyr Leu Ser Leu Pro His Lys Asn Lys Val Pro Trp Asn Arg Leu  
GGT ATT CTT GGA TCT CCA TCA CAG GAA GAT CTG AAT TGT ATA ATA AAT TTA AAA OCT ACA AAT TAT TTG CTT TCT CTC CGC CAC AAA AAT MAG GTG CGC TGG AAC AGG TTG 999

Phe Pro Asn Ala Asp Ser Lys Ala Leu Asp Leu Leu Thr Phe Asn Pro His Lys Arg Ile Glu Val Gln Ala Leu Ala His Pro Tyr Leu Glu Gln  
TTC CCA AAC GGT GAC TCC AAA GCT CTG GAT TTA CTG CAT AAA ATG TTG ACA TTT AAC CCT CAC AAG AGG ATT GAA GTT GAA CAG GCT CTG GCC CAC CGC TAC CTG GAG CAG 1110

Tyr Tyr Asp Pro Ser Asp Glu Pro Ile Ala Glu Ala Pro Phe Lys Phe Asp Met Glu Leu Asp Asp Leu Pro Lys Glu Lys Leu Lys Glu Leu Ile Phe Glu Thr Ala  
TAT TAT GAC CCA AGT GAT CAG CCT ATT GCT GAA CCA CCA TTC AAG TTT GAC ATG GAG CTG GAC TTA CCT AAG CAG ARG CTC AAA GAA CTC ATT TTT GAA CAG ACT GCT 1221

Arg Phe Gln Pro Gly Tyr Arg Ser END  
CBA TTC CAG CCA GGA TAC AGA TCT TAA ATTGGTCAGGACAGAGCGCTCAGAGACTGGAGCGCTTCAGATCGGTGTGCCGCCAGTTCTTAGCCCTGCTGTCTCCAGCCGTCTCAGCTTCCACTCTTCGACTC 1359

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FIGURE 3A

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FIGURE 3B







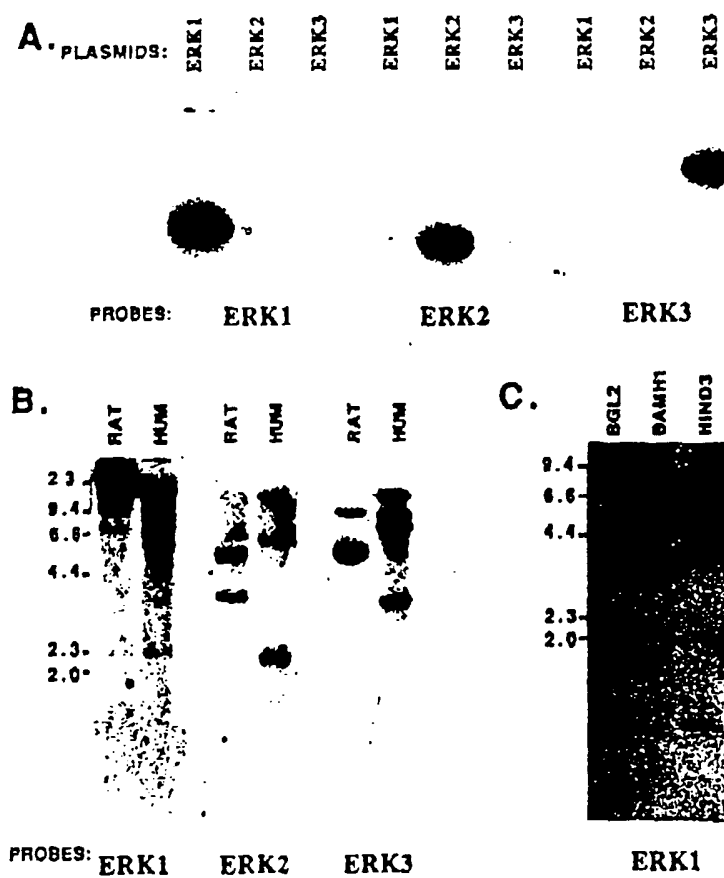


FIGURE 5

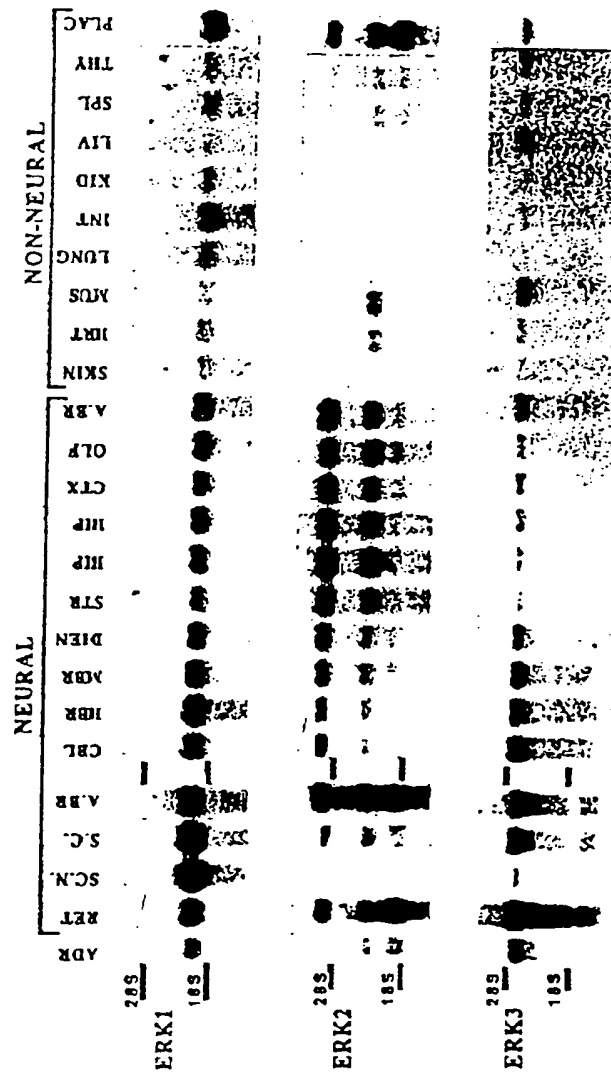


FIGURE 6A

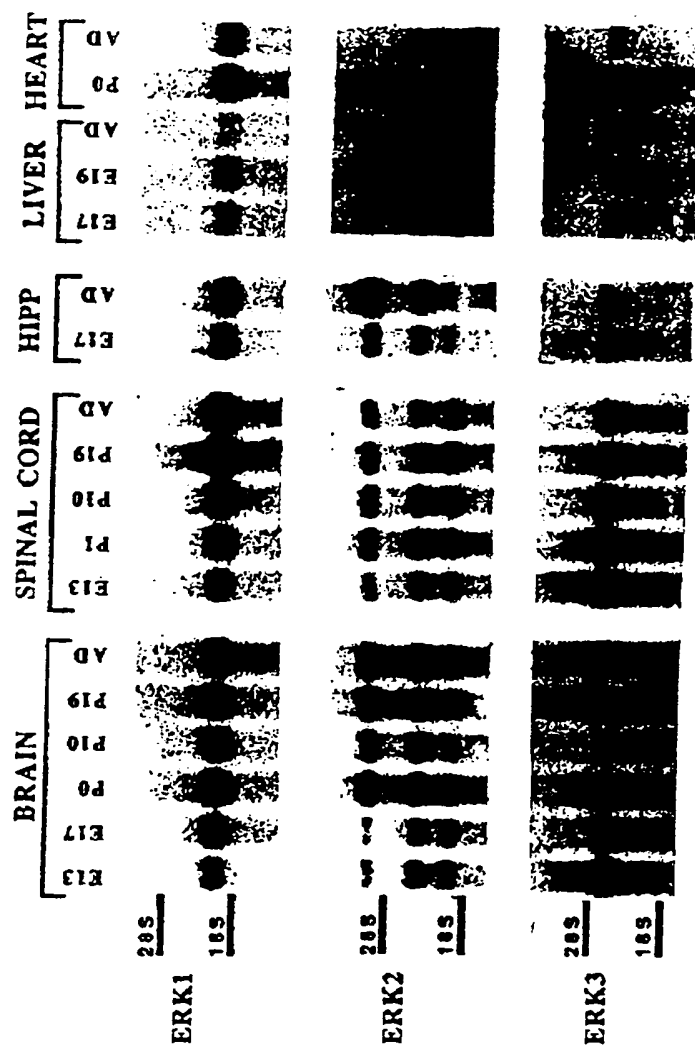


FIGURE 6B

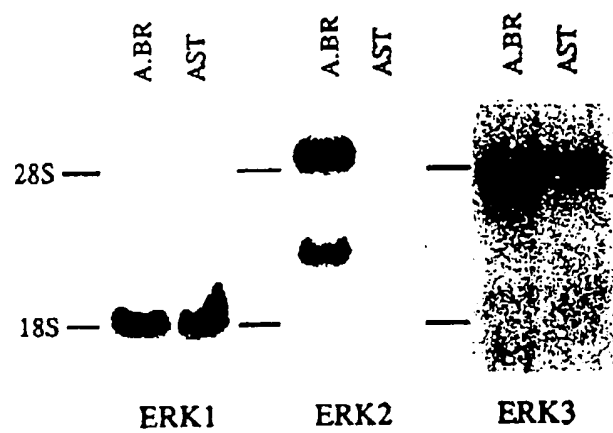


FIGURE 6C

12/18

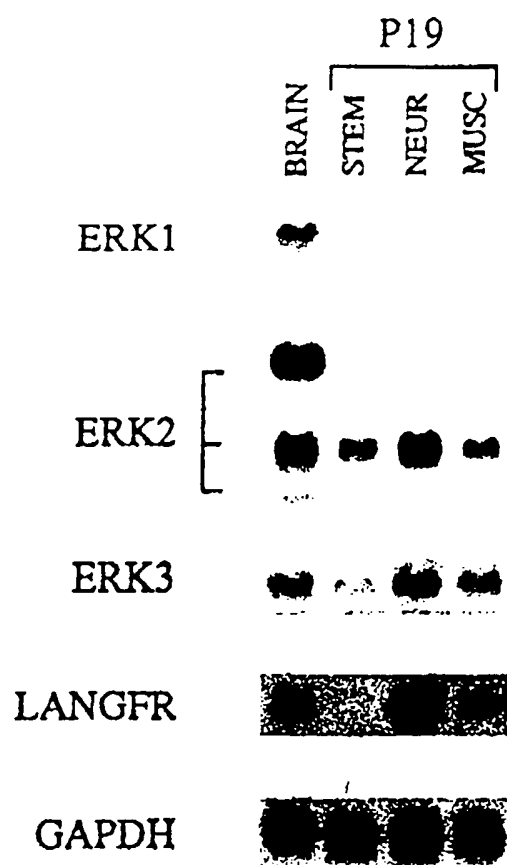


FIGURE 6D

# FIGURE 7

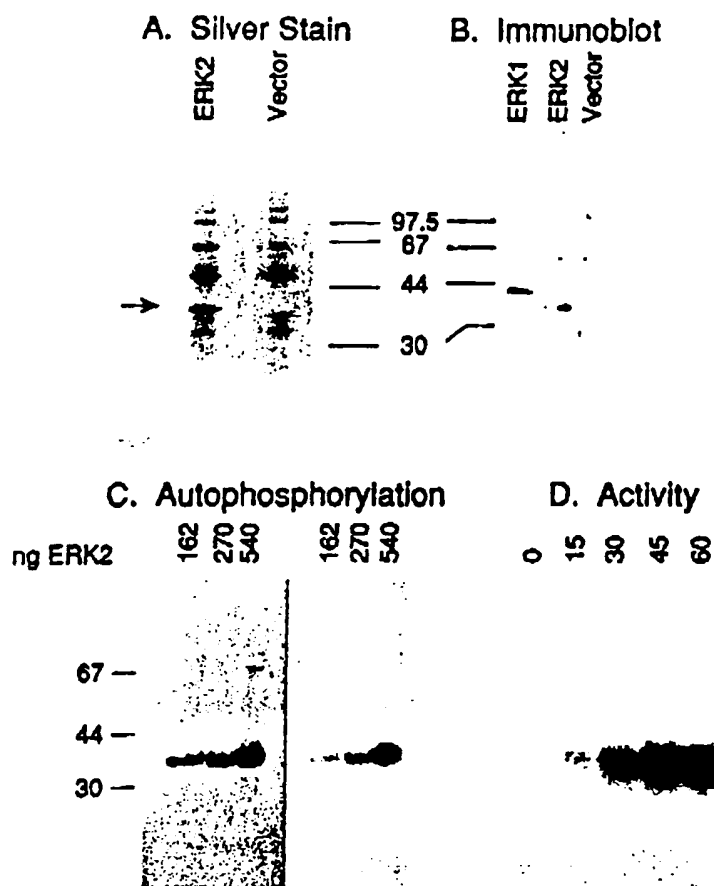


FIGURE 8

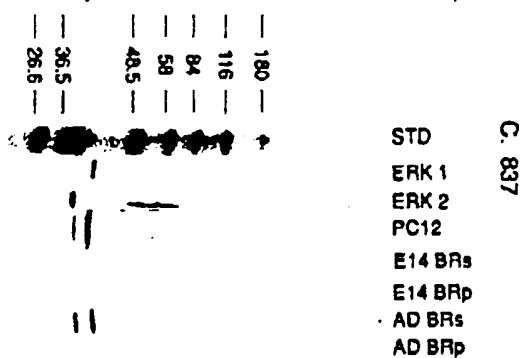
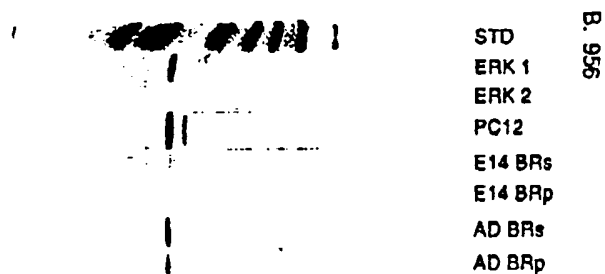
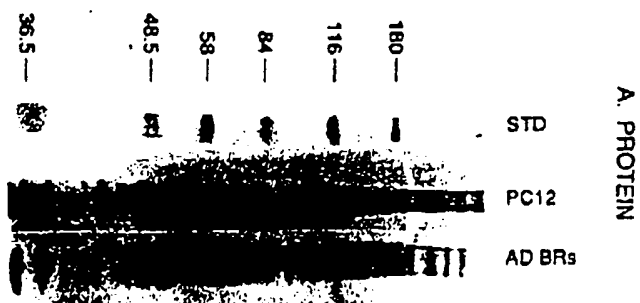


FIGURE 9 A-B

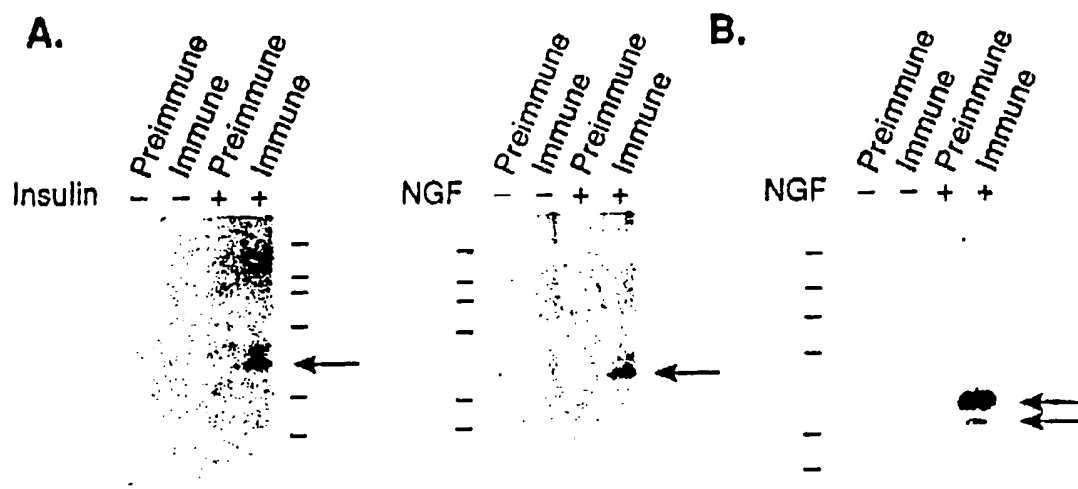




FIGURE 9C

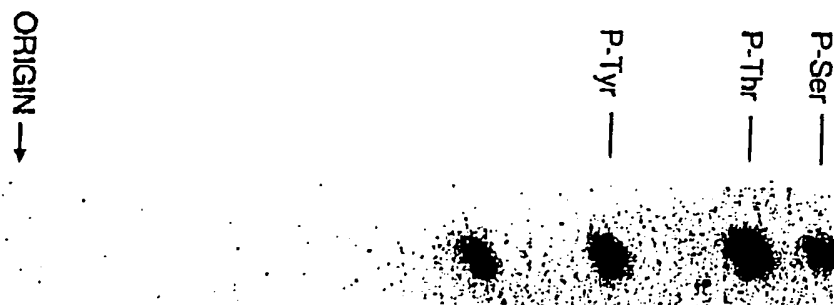
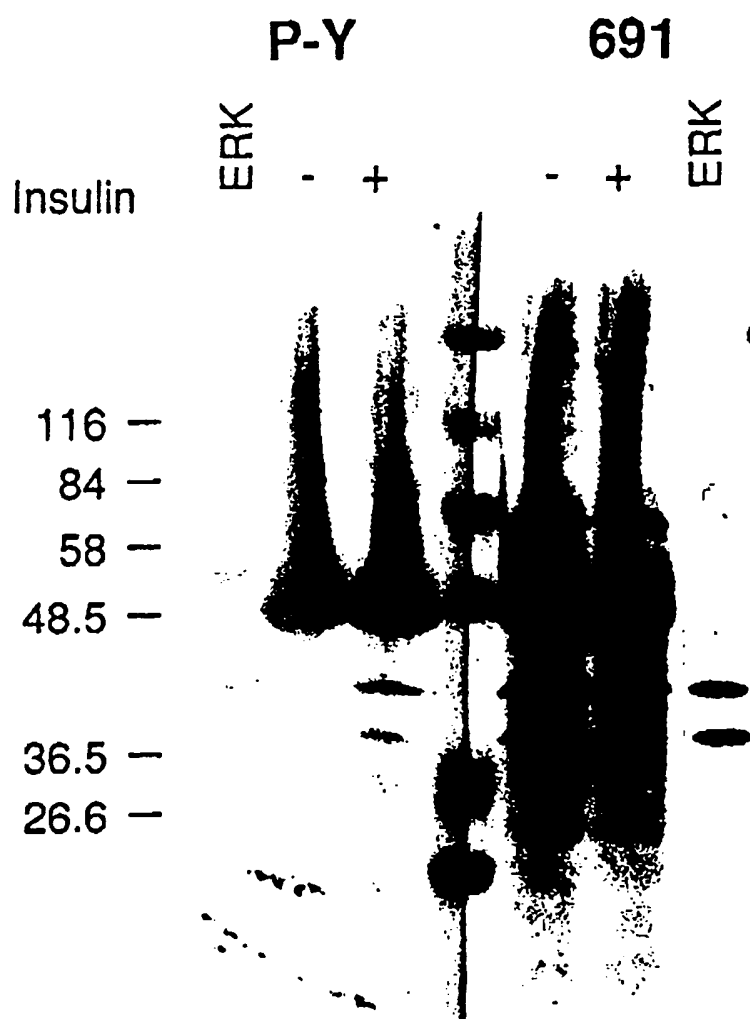


FIGURE 10





# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/03894

|   |   |   |
|---|---|---|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) *  |   |   |
| According to International Patent Classification (IPC) or to both National Classification and IPC<br>I.P.C. (5): C12Q 1/68, C07H 15/12; C07K 3/00; C12N 15/00<br>U.S. CL: 435/6; 536/27; 935/77, 78; 530/350  |   |   |
| <b>II. FIELDS SEARCHED</b>  |   |   |
| Minimum Documentation Searched *  |   |   |
| Classification System   | Classification Symbols  |   |
| US. CL.   | 435/6, 536/27; 935/77, 78; 530/350  |   |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *   |   |   |
| GENBANK, EMBL   |   |   |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>   |   |   |
| Category *  | Citation of Document, ** with indication, where appropriate, of the relevant passages **  | Relevant to Claim No. **  |
| Y, P  | Science. Vol. 249. issued July 1990.<br>Boulton et al., "An Insulin-Stimulated Protein Kinase Similar to Yeast Kinases involved in Cell Cycle Control", pages 64-67, see especially Figs. 1 and 3.              | 1-45  |
| X<br>Y  | Cell. vol. 58, issued 22 September 1989,<br>Courchesne et al., "A Putative Protein Kinase overcomes Pheromone-Induce Arrest of Cell Cycling in <i>S. cerevisiae</i> " page 1107-1119, see especially Figs. 293. | 1-6, 10,<br>17-21, 28-<br>31, 34<br>7-9, 11-16<br>22-28, 32-<br>33, 35-45 |
| <p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> |   |   |
| <b>IV. CERTIFICATION</b>  |   |   |
| Date of the Actual Completion of the International Search   | Date of Mailing of this International Search Report   |   |
| 22 September 1991   | 28 OCT 1991   |   |
| International Searching Authority   | Signature of Authorized Officer   |   |
| ISA/US  | Mindy B. Fleisher<br>Mindy B. Fleisher TF   |   |